# TOWARDS THE DEVELOPMENT OF TRANSGENIC *BANANA BUNCHY TOP VIRUS* (BBTV)-RESISTANT BANANA PLANTS: INTERFERENCE WITH REPLICATION

BY

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A thesis submitted for the degree of Doctor of Philosophy in the Centre for Tropical Crops and Biocommodities, Queensland University of Technology, Australia

**2008**

# ABSTRACT

*Banana bunchy top virus* (BBTV) causes one of the most devastating diseases of banana. Transgenic virus resistance is now considered one of the most promising strategies to control BBTV. Pathogen-derived resistance (PDR) strategies have been applied successfully to generate plants that are resistant to numerous different viruses, primarily against those viruses with RNA genomes. BBTV is a circular, single-stranded (css) DNA virus of the family *Nanoviridae*, which is closely related to the family *Geminiviridae*. Although there are some successful examples of PDR against geminiviruses, PDR against the nanoviruses has not been reported. Therefore, the aim of this thesis was to investigate the potential of BBTV genes to interfere with virus replication when used as transgenes for engineering banana plants resistance to BBTV. The replication initiation protein (Rep) of nanoviruses is the only viral protein essential for viral replication and represents an ideal target for PDR. Therefore, this thesis focused on the effect of wild-type or mutated Rep genes from BBTV satellite DNAs or the BBTV integral genome on the replication of BBTV in banana embryogenic cell suspensions.

A new Rep-encoding satellite DNA, designated BBTV DNA-S4, was isolated from a Vietnamese BBTV isolate and characterised. When the effect of DNA-S4 on the replication of BBTV was examined, it was found that DNA-S4 enhanced the replication of BBTV. When the replicative capabilities of DNA-S4 and the previously characterised Rep-encoding BBTV satellite, DNA-S1, were compared, it was found that the amount of DNA-S4 accumulated to higher levels than DNA-S1. The interaction between BBTV and DNA-S1 was also examined. It was found that over-expression of the Rep encoded by DNA-S1 using *ubi*1 maize polyubiquitin promoter enhanced replication of BBTV. However, when the Rep-encoded by DNA-S1 was expressed by the native S1 promoter (in plasmid pBT1.1-S1), it suppressed the replication of BBTV. Based on this result, the use of DNA-S1 as a possible transgene to generate PDR against BBTV was investigated.

The roles of the Rep-encoding and U5 genes of BBTV DNA-R, and the effects of over-expression of these two genes on BBTV replication were also investigated. Three mutants of BBTV DNA-R were constructed; plasmid pUbi-RepOnly-nos contained the *ubi*1 promoter driving Rep expression from DNA-R, plasmid pUbi-IntOnly-nos contained the *ubi*1 promoter driving expression of the DNA-R internal gene product (U5), while plasmid pUbi-R.ORF-nos contained the *ubi*1 promoter driving the expression of both Rep and the internal U5 gene product. The replication of BBTV was found to be significantly suppressed by pUbi-RepOnly-nos, weakly suppressed by pUbi-IntOnly-nos, but strongly enhanced by pUbi-R.ORF-nos.

The effect of mutations in three conserved residues within the BBTV Rep on BBTV replication was also assessed. These mutations were all made in the regions in the ATPase motifs and resulted in changes from hydrophilic to hydrophobic residues (i.e. K187→M, D224→I and N268→L). None of these Rep mutants was able to initiate BBTV replication. However, over-expression of Reps containing the K187→M or N268→L mutations significantly suppressed the replication of BBTV.

In summary, the Rep constructs that significantly suppressed replication of DNA-R and -C in banana embryogenic cell suspensions have the potential to confer resistance against BBTV by interfering with virus replication. It may be concluded that BBTV satellite DNAs are not ideal for conferring PDR because they did not suppress BBTV replication consistently. Wild-type Rep transcripts and mutated (i.e. K187→M and N248→L) Rep proteins of BBTV DNA-R, however, when over-expressed by a strong promoter, are all promising candidates for generating BBTV-resistant banana plants.

**Keywords:** *Banana bunchy top virus*; nanoviruses; replication initiation protein (Rep); pathogen-derived resistance; BBTV DNA-R; BBTV DNA-S1; BBTV DNA-S3; BBTV DNA-S4; satellite DNA; ATPase; post-transcriptional gene silencing; RNA silencing suppressor.

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# List of Abbreviations

| | |
|---|---|
| aa | amino acid(s) |
| AAA+ ATPase | ATPase associated with various cellular activities |
| ABTV | Abaca bunchy top virus |
| ACMV | African cassava mosaic virus |
| ADK | adenosine kinase |
| AGO | Argonaute |
| ATP-A | ATPase motif A |
| ATP-B | ATPase motif B |
| ATP-C | ATPase motif C |
| BBTD | Banana bunchy top disease |
| BBTV | Banana bunchy top virus |
| BDMV | Bean dwarf mosaic virus |
| BGMV | Bean golden mosaic virus |
| BGYMV | Bean golden yellow mosaic virus |
| BNYVV | Beet necrotic yellow vein virus |
| CabLCV | Cabbage leaf curl virus |
| CFDV | Coconut foliar decay virus |
| CLCuV | Cotton leaf curl virus |
| Clink | cell-cycle link protein |
| CP | capsid (or coat) protein |
| CR-M | major common region |
| CR-SL | stem-loop common region |
| css | circular, single-stranded |
| DCL | Dicer-like enzyme |
| DI | defective interfering |
| DNA | deoxyribonucleic acid |
| ds | double-stranded |
| EACMCV | East African cassava mosaic Cameroon virus |
| FBNYV | Faba bean necrotic yellows virus |
| HYVV | Huasteco yellow vein virus |

| | |
|---|---|
| kb | kilobase(s) |
| M-Rep | master Rep |
| MDV | Milk vetch dwarf virus |
| miRNA | microRNA |
| MP | movement protein |
| mRNA | messenger RNA |
| MYMV-Vig | Mungbean yellow mosaic virus-Vigna |
| NSP | nuclear-shuttle protein |
| nt | nucleotide(s) |
| NTP | nucleoside-triphosphate |
| nm | nanometre(s) |
| ORF | open-reading frame |
| PCNA | proliferative cell nuclear antigen |
| PCR | polymerase chain reaction |
| PDR | pathogen-derived resistance |
| PolyA | polyadenylation |
| pRBR | plant Rb-binding protein |
| PTGS | post-transcriptional gene silencing |
| PYMV | Potato yellow mosaic virus |
| Rb | retinoblastoma |
| RCR | rolling-circle replication |
| RCR-1 | rolling-circle replication motif 1 |
| RCR-2 | rolling-circle replication motif 2 |
| RCR-3 | rolling-circle replication motif 3 |
| RDR | RNA-dependent RNA polymerase |
| Rep | replication initiation protein |
| RISC | RNA-induced silencing complex |
| RNA | ribonucleic acid |
| RNAi | RNA interfering molecule(s) |
| SCSV | Subterranean clover stunt virus |
| SF3 | superfamily 3 |
| siRNA | short interfering RNA |
| SLCMV | Sri Lankan cassava mosaic virus |
| ss | single-stranded |
| T-Rep | truncated Rep without the ATPase domain |
| TGMV | Tomato golden mosaic virus |

| | |
|---|---|
| TGS | transcriptional gene silencing |
| ToLCNDV | Tomato leaf curl New Delhi virus |
| ToLCV | Tomato leaf curl virus |
| ToMoV | Tomato mottle virus |
| TYLCD | Tomato yellow leaf curl disease |
| TYLCV | Tomato yellow leaf curl virus |

# STATEMENT OF ORIGINAL AUTHORSHIP

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signed_____ Date_____

Theresa Tsao

# ACKNOWLEDGEMENTS

I would like to thank my supervisors Rob Harding, James Dale and Marion Bateson for providing me with invaluable advice and understanding.

I would also like to thank the past and present members of the plant group, especially Scott Hermann, Doug Becker, Katherine Bell, Don Catchpoole, Cathryn Horser, Jennifer Kleidon, Peter Revill and Matthew Webb.

Finally, I would like to thank my parents, Juliana and Herbert, for their love and support, without whom, I would not have got thus far.

# CHAPTER 1

## AIMS AND OBJECTIVES

## Description of Scientific Problem Investigated

Banana bunchy top disease, caused by *Banana bunchy top virus* (BBTV), is considered the most devastating virus disease of banana (*Musa* spp.). BBTV is a circular, single-stranded (css) DNA virus classified in the genus *Babuvirus*, within the family *Nanoviridae*. The cssDNA genome of BBTV comprises at least six integral components. Like the closely-related geminiviruses, BBTV replicates by a master replication initiation protein (M-Rep)-mediated rolling-circle type mechanism. BBTV DNA-R encodes the master Rep (M-Rep) that directs self replication in addition to replication of other BBTV genome components. Additional Rep-encoding cssDNAs, termed satellite DNAs, have also been associated with some BBTV isolates; these components are not considered integral genomic components because, although capable of self-replication, the encoded Rep is unable to direct the replication of integral BBTV components.

While partial control of BBTV has been achieved in Australia via the implementation of strict phytosanitary protocols, this approach is expensive and labour intensive. Furthermore, generating BBTV-resistant banana plants by conventional breeding is not possible due to the problems of sterility with commercially significant banana varieties and a lack of sources of BBTV-resistance. Molecular breeding is now considered the most promising strategy to develop BBTV-resistant bananas. The research in this thesis investigated strategies that may interfere with BBTV replication and that could be exploited subsequently to generate BBTV-resistant transgenic banana plants.

## Overall Objectives of the Study

Molecular breeding, based on pathogen-derived resistance (PDR) strategies, has been used with great success against many RNA viruses but there have been considerably fewer reports of PDR used against DNA viruses and no reports of PDR used against nanoviruses. Studies using the closely-related geminiviruses have shown that the most effective transgene for PDR is the gene that encodes the replication initiation protein (Rep).

As the BBTV Rep is the only viral gene essential for replication, this gene was considered an ideal target for the development of transgenic resistance. The choice of Rep as a target was also based on previous studies in our laboratory that showed that the Rep encoded by BBTV satellite DNA-S1 suppressed the replication of BBTV. Based on these observations, the objective of this study was to investigate the use of a variety of different Rep gene-related strategies to interfere with BBTV replication and that could possibly be exploited to generate BBTV-resistant banana plants.

## Specific Aims of the Study

The specific aims of this thesis were (i) to confirm the suppressive effect of the BBTV satellite DNA-S1-encoded Rep on replication of integral BBTV components, (ii) to examine the effect of a different BBTV satellite DNAs on BBTV replication and (iii) to examine the effect of over-expression of wild-type and mutated BBTV M-Rep on the replication of the BBTV integral genome in banana embryogenic cell suspensions.

## Account of Scientific Progress Linking the Chapters

Previous preliminary research in our laboratory showed that BBTV satellite DNA-S1 suppressed replication of BBTV in banana embryogenic cell suspensions. In **Chapter 3** of this thesis, studies on the effect of BBTV satellite DNAs on BBTV replication were undertaken. Initially, previous studies that examined the suppressive effect of DNA-S1 were repeated using a larger data set and using an extended experimental time frame. These results confirmed the previous findings and, based on this outcome, banana embryogenic cell suspensions were transformed with BBTV DNA-S1 constructs. To increase the level of DNA-S1 mediated suppression on BBTV replication, experiments were conducted to examine the effect of over-expression of the Rep encoded by DNA-S1. Finally, to determine the effect of other BBTV satellite DNAs on BBTV replication, an additional BBTV satellite DNA, designated DNA-S4, was cloned, sequenced and characterised from a Vietnamese BBTV isolate. Unlike results obtained using DNA-S1, use of DNA-S4 resulted in an unexpected enhancement of BBTV replication in banana cell suspensions.

In **Chapter 4,** the effect of over-expression of ORFs of BBTV M-Rep on BBTV replication was examined. BBTV DNA-R constructs were generated that contained mutations to (i) the major Rep-encoding ORF only and (ii) the smaller, internal ORF (U5) only, that rendered these ORFs untranslatable. A further control construct was made that contained both translatable ORFs of wild-type BBTV DNA-R. In all constructs, gene expression was controlled by the strong constitutive maize polyubiquitin promoter. These studies showed that replication of BBTV was (i) strongly suppressed in the presence of the construct containing the untranslatable U5 ORF and (ii) weakly suppressed in the presence of the construct containing the U5 ORF alone (without the Rep ORF). Further, over-expression of both DNA-R ORFs resulted in an enhancement of BBTV replication.

In the final experimental chapter (**Chapter 5),** the effect of three site-specific mutations, K187→M, D221→I and N248→L, to the BBTV M-Rep on virus replication was examined. The K187, D221 and N248 are conserved amino acids found in the ATPase motifs of the BBTV M-Rep proteins. It was found that all three Rep mutants were unable to initiate BBTV replication, and that the over-expression of two Rep mutants (K187→M and N248→L) significantly suppressed BBTV replication in cell suspensions.

# CHAPTER 2

# LITERATURE REVIEW

# *2.1. Banana bunchy top virus*

Banana is the common name for plants in the genus *Musa* of the family *Musaceae*. Banana is one of the most important world crops and is mainly cultivated for its fruit. Annual production of banana fruit exceeds 70 million tonnes (FAOSTAT, 2007). The most economically important disease of banana is banana bunchy top disease (BBTD), caused by *Banana bunchy top virus* (BBTV). Plants infected by BBTV may completely fail to produce fruit.

## *2.1.1. Symptoms*

The most distinctive symptom of BBTD is the characteristic "morse-code" pattern that is formed by dark green streaking of veins in the leaf lamina, midribs and petioles, or dark red streaks in the floral bracts (Darnell-Smith, 1924). Darker streaks are comprised of chlorophyllous parenchyma cells that are located near the sieve tubes in (or surrounding) the phloem (Darnell-Smith, 1924). BBTV induces hypertrophy of unspecialised cells present between the parenchyma cells and sieve tubes of the phloem, making these unspecialised cells (and their nuclei) larger than normal (Magee, 1939). These abnormalities may lead to obliteration and necrosis of the phloem (Magee, 1939). Leaf margins may become chlorotic because the distorted phloem fails to transport essential assimilates efficiently (Magee, 1939). Instead of spreading normally, the leaves and leaf lamina of infected plants grow upright to form the characteristic rosette appearance that gives the name "bunchy top" to the disease (Darnell-Smith, 1924).

Infections caused by BBTV can be primary or secondary (Magee, 1927). Primary infections occur when a banana plant arises from infected planting

material. Primary-infected plants have more severe and more distinctive symptoms - these plants are dwarfed, usually smaller than 1 m in height and they may not produce any fruit. Secondary infection of plants occurs by aphid transmission after an initial period of BBTV-free plant growth. In secondary-infected plants, symptoms are milder and only appear in tissues formed after the infection. Early stages of the secondary infection are often confused with signs of nutrient deficiency or environmental stress (Darnell-Smith, 1919; Darnell-Smith and Tryon, 1923). An average of 25 days incubation period is usually required for development of symptoms, although symptoms may develop more rapidly in warmer temperatures (Magnaye and Valmayor, 1995; Dale *et al.,* 1986).

### *2.1.2. Host range*

BBTV infects *Musa acuminata*, *M. balbisiana*, *M. acuminata x balbisiana*, *M. coccinea*, *M. jackeyi*, *M. ornata*, *M. textilis and M. velutina* (Magee, 1927; 1948; Espino *et al.,* 1993; Thomas and Iskra-Caruana, 2000; Thomas and Dietzgen, 1991; Furuya *et al.,* 2003). BBTV also infects *Ensete ventricosum* (Welw.) E.E. Cheesm (syn. *Musa ensette*), a closely related species in the *Musaceae* (Wardlaw, 1961). Outside the *Musaceae*, the species *Alpinia purpurata* (red ginger) and *Colocasia esculenta* (L) (taro) in India, and *Canna indica* (Canna) and *Hedychium coronarium* (garland flower) in Taiwan, have been reported as alternative hosts for BBTV, although, these species have not been found as hosts for BBTV in Australia or Hawaii and this requires clarification (Ram and Summanwar, 1984; Su *et al.,* 1993; Geering and Thomas, 1991; Hu *et al.,* 1996).

### *2.1.3. Susceptibility*

No species, cultivar, type or variety in the genus *Musa* has been found to be completely immune to BBTV. The dessert cultivar Gros Michel[1] showed some resistance in field observations in Australia, Fiji and Northern Borneo; however, with prolonged incubation, the plant eventually displayed typical severe symptoms (Magee, 1948; Muharam, 1984). Kanchikela and Venattukunnan[2] cultivars in India also showed some level of BBTV resistance with an infection rate of less than 10 % (Jose, 1981). Low levels of resistance have also been observed in Jimbluk[3], Klutuk[4], Kapas and Seribu[5] cultivars (Sulyo, 1992). The Fijian cultivar Veimama[6] has been reported to recover partially from severe symptoms and to produce healthy foliage and a bunch, but the "morse-code" pattern remained on almost every leaf (Magee, 1948).

### *2.1.4. Virus transmission*

Plants with BBTD symptoms were first recognised in Fiji in 1889, and subsequently in many other regions of the world, except Latin America and the Caribbean (Jones, 1993; Thomas and Iska-Caruana, 2000; Kagy *et al.*, 2001). The international spread of BBTV is primarily *via* distribution of infected planting material such as suckers and corms (Wardlaw, 1961).

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1 Gros Michel is a dessert cultivar of the Cavendish variety. It has the AAA genome, which refers to 3n karyotype originated entirely from *M. acuminata.*

2 Kanchikela and Venattukunnan are cooking banana varieties of the Bluggoe variety. Bluggoe banana has the ABB genome, with one set of chromosomes from *M. balbisiana* and two sets of chromosomes from *M. acuminata.*

4 Ploidy of variety Jimbluk is unknown.

4 Klutuk is a variety of *M. balbisiana* and has the BB genome.

5 Cultivars, Kapas and Seribu, both have the AAB genome.

6 Veimama is a dessert cultivar with the AAA genome.

The banana aphid (*Pentalonia nigronervosa*) is responsible for local transmission of BBTV. It is an efficient insect vector that may have co-evolved with bananas in Southeast Asia (Magnaye and Valmayor, 1995). Although banana aphids mainly colonise bananas, they have also been found on related genera including *Heliconia*, *Strelitzia* and *Ravenala* (Magnaye and Valmayor, 1995). Brief periods of banana aphid colonisation have also been observed on the genus *Canna* and *Zingiber* (Magnaye and Valmayor, 1995). For banana aphids to become infective, at least 12 hours of feeding on BBTV-infected plants is required (Magee, 1948; Quebral, 1963; Hafner *et al.,* 1995; Hu *et al.,* 1996). To transmit the virus, infective banana aphids need at least 1.5 hours of feeding on susceptible plants (Quebral, 1963). The virus remains in the aphid vector after moulting, but is not passed to the insect progeny (Magnaye and Valmayor, 1995).

### *2.1.5. Taxonomy*

BBTV is the type species of the genus *Babuvirus* in the family *Nanoviridae* (Büchen-Osmond, 2007). Abaca bunchy top virus (ABTV) is the other species in the genus *Babuvirus* (Sharman *et al.,* 2007). Both BBTV and ABTV infect plant species in the genus *Musa*, and both viruses consist of six circular, single-stranded (css) DNA components, DNA-R, -U3, -S, -M, -C and -N, in the integral genome (Sharman *et al.,* 2007). There are a few differences however, between the two viruses. For example, BBTV DNA-R contains a second ORF (i.e. a small open reading frame (ORF) located internal to the Rep-encoding ORF, whereas ABTV DNA-R only contains the Rep-encoding ORF (Sharman *et al.,* 2007). In addition, BBTV DNA-U3 encodes the U3 ORF, but ABTV DNA-U3 does not have any ORF (Sharman *et al.,* 2007).

The family *Nanoviridae* (nanoviruses) also contains the genus *Nanovirus*, which contains *Faba bean necrotic yellows virus* (FBNYV), *Milk vetch dwarf virus* (MDV) and *Subterranean clover stunt virus* (SCSV). Nanoviruses are all transmitted by aphid vectors (Büchen-Osmond, 2007). Genomes of nanoviruses are all replicated by a rolling-circle mechanism mediated by the viral replication initiation protein (Rep) (Büchen-Osmond, 2007). Their genomes all consist of multiple cssDNA molecules, approximately 1 kb in size which usually encodes a single protein (Büchen-Osmond, 2007).

There are a few differences between the genus *Babuvirus* and *Nanovirus*. Firstly, the cssDNAs of the viruses in the genus *Nanovirus* each contain only a single ORF (Büchen-Osmond, 2007). Secondly, viruses in the genus *Babuvirus* all infect *Musa* spp. (monocotyledon), but viruses in the genus *Nanovirus* infect only dicotyledonous plants (Büchen-Osmond, 2007; Sherman *et al.,* 2007). Thirdly, the integral genome of viruses in the genus *Babuvirus* consist of at least six cssDNA molecules, but viruses in the genus *Nanovirus* have a genome consisting of at least eight cssDNA molecules (Vetten *et al.,* 2005).

Members of the family *Geminiviridae* (geminiviruses) are also cssDNA viruses that infect plants and are replicated by a Rep-mediated rolling circle mechanism (Büchen-Osmond, 2007). The genome of geminiviruses consists however, of only one or two cssDNA molecules, and each cssDNA encodes multiple ORFs. Geminiviruses are also distinct from nanoviruses in morphology, mode of transcription, host plants and vector species.

### *2.1.6. Organisation of the BBTV integral genome*

The integral genome of BBTV comprises at least six cssDNA molecules that are found consistently in all geographical isolates of the virus (Karan *et al.,* 1994; 1997). Each of these cssDNAs is approximately 1 kb in length and is individually encapsidated in icosahedral virions 18-20 nm in diameter (Burns *et al.,* 1995) (Fig.2-1). These cssDNAs were initially known as BBTV DNA-1 to -6, but recently were renamed BBTV DNA-R, -U3, -S, -M, -C and -N, respectively, to better represent the functions of their encoded proteins (Vetten *et al.,* 2005). The sizes and functions of the six cssDNAs are summarised in Table 2-1.

The BBTV genome encodes all of the proteins on the virion sense. BBTV DNA-R encodes two ORFs, one internal to the other. The large ORF of DNA-R encodes a Rep (Hafner *et al.,* 1997b). The Rep is the best characterised protein of BBTV; its various functions are detailed later. The small internal U5 ORF of DNA-R has an unknown function, but it has been speculated that the gene product may have a role in regulating expression of Rep (Beetham *et al.,* 1997). The function of DNA-U3 is unknown, while DNA-S encodes the viral coat protein (CP) (Beetham *et al.,* 1999). DNA-M encodes a putative movement protein (MP) with a hydrophobic N-terminus (Wanitchakorn *et al.,* 1997). DNA-C encodes a protein that presumably facilitates viral replication by switching the plant host cells into S-phase. This protein has the LxCxE motif that can be found in typical Clink (cell-cycle link) proteins that bind with plant retinoblastoma (Rb)-like proteins (Wanitchakorn *et al.,* 2000). DNA-N encodes a nuclear shuttle protein (NSP) (Wanitchakorn *et al.,* 2000). The minimum infectious genome unit required to induce typical BBTD symptoms has yet to be determined.

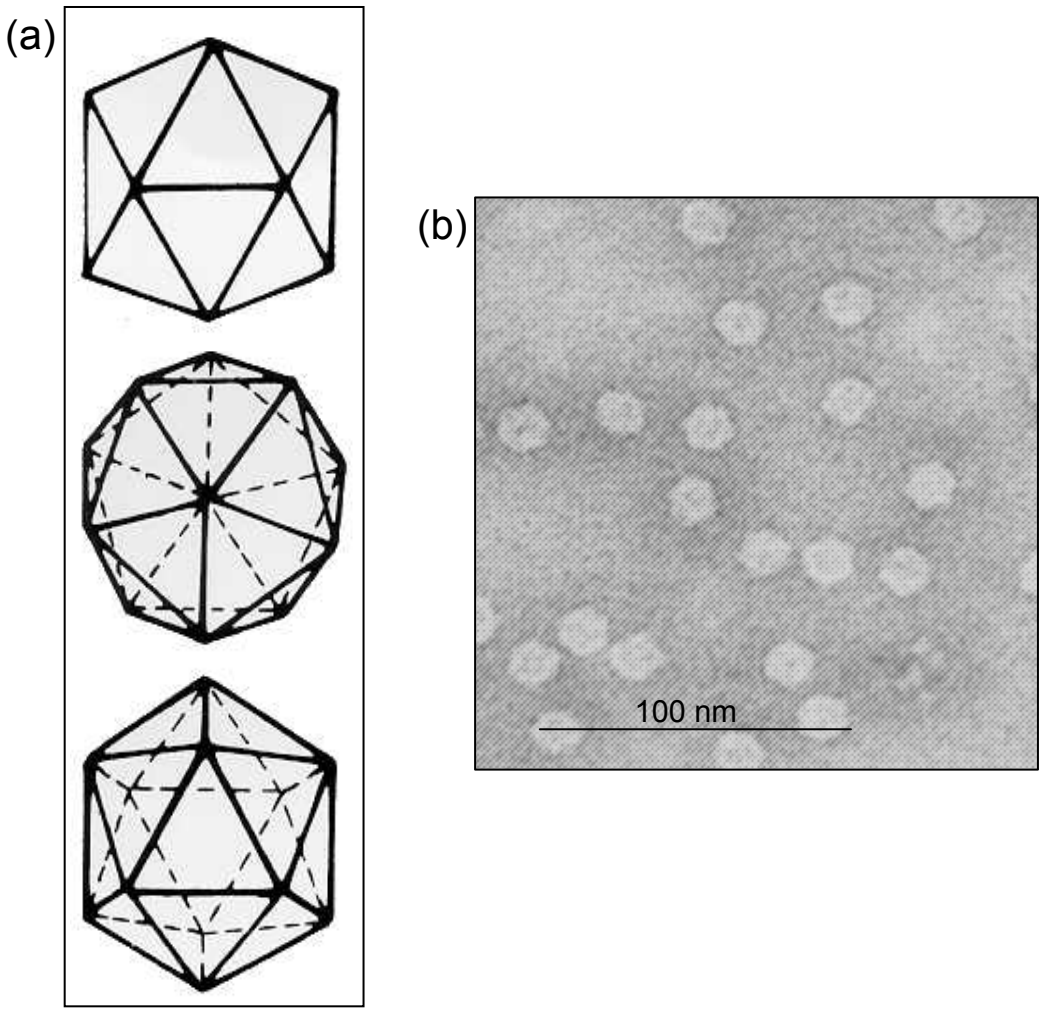



**Fig. 2-1. Virions of BBTV are regular icosahedrons.**

(a) An icosahedron is composed of 20 triangular faces and 30 edges. The figure shows the icosahedron viewed from different angles to the axis.

(b) An electron micrograph (Harding *et al.,* 1991) of BBTV purified by centrifugation in a caesium sulphate gradient and stained with 2% ammonium molybdite, pH 6.5.

**Table 2-1. The proteins encoded by the integral genome components of BBTV.**

| *Name* | *Size* of DNA Component (nt)* | *ORF Size (nt)* | *Function of Encoded Protein* | *Protein Size* (kDa)* |
|---|---|---|---|---|
| **DNA-R** | 1111 | 861 | **Replication Initiation Protein (Rep)** | 33.6 |
| | | 126 | ***unknown function* (U5)** | 5 |
| **DNA-U3** | 1060 | 251 | ***unknown function* (U3)** | 10 |
| **DNA-S** | 1075 | 525 | **Capsid/Coat Protein (CP)** | 20 |
| **DNA-M** | 1043 | 351 | **Movement Protein (MP)** | 14 |
| **DNA-C** | 1018 | 483 | **Cell-Cycle Link Protein (Clink)** | 19 |
| **DNA-N** | 1089 | 462 | **Nuclear-Shuttle Protein (NSP)** | 17 |

**These sizes are of BBTV Australian isolate.*

The intergenic region of the six integral BBTV genome components share two highly conserved regions that are known as the stem-loop common region (CR-SL) (Fig. 2-2a) and the major common region (CR-M). The CR-SL is a 69 nucleotide (nt) sequence with 62% identity among the BBTV integral genome components (Hafner, 1998). This 69 nt sequence contains (1) a 20 nt base-paired stem, within which 14 nt are fully conserved in all BBTV cssDNA components and (2) an 11 nt loop, within which 9 nt (TAnTATTAC[7]) are conserved in all nanoviruses and geminiviruses (Hafner, 1998). A 13 nt sequence, that is located downstream (3') of the stem-loop structure, is also highly conserved in the BBTV genome (Hafner, 1998). These 13 nt consist of the virion-sense (forward) F1 and F2 iterons (GGGAC), located adjacent to each other as tandem repeats. The F1 and F2 iterons are 2 nt downstream of the stem-loop of BBTV DNA-R, -S, -M, -C and -N, and 1 nt downstream of the stem-loop of BBTV DNA-U3 (Horser, 2000). A conserved G-box ([CG]ACGTA[8]) is located immediately upstream of the stem-loop. A single anti-sense (reverse) iteron (GTCCC), designated R, is also found 19 (DNA-R, -S, -M, -C), 90 (DNA-U3) or 10 (DNA-N) nt upstream of the stem-loop. The iterons are thought to play a major role in the specific interaction between the Rep and the cssDNA genome components during replication (Horser, 2000; Herrera-Valencia *et al.*, 2006).

The CR-M is located 20-233 nt upstream of the CR-SL. The CR-M is generally a 65 to 92 nt sequence with 76 % homology among the BBTV integral genome components (Hafner, 1998). The 3' end of the CR-M has a 15 nt G-C rich

7 The n indicates any residue.

8 The [ ] indicates that any one of the given residues may be found in this position of the aligned sequences.

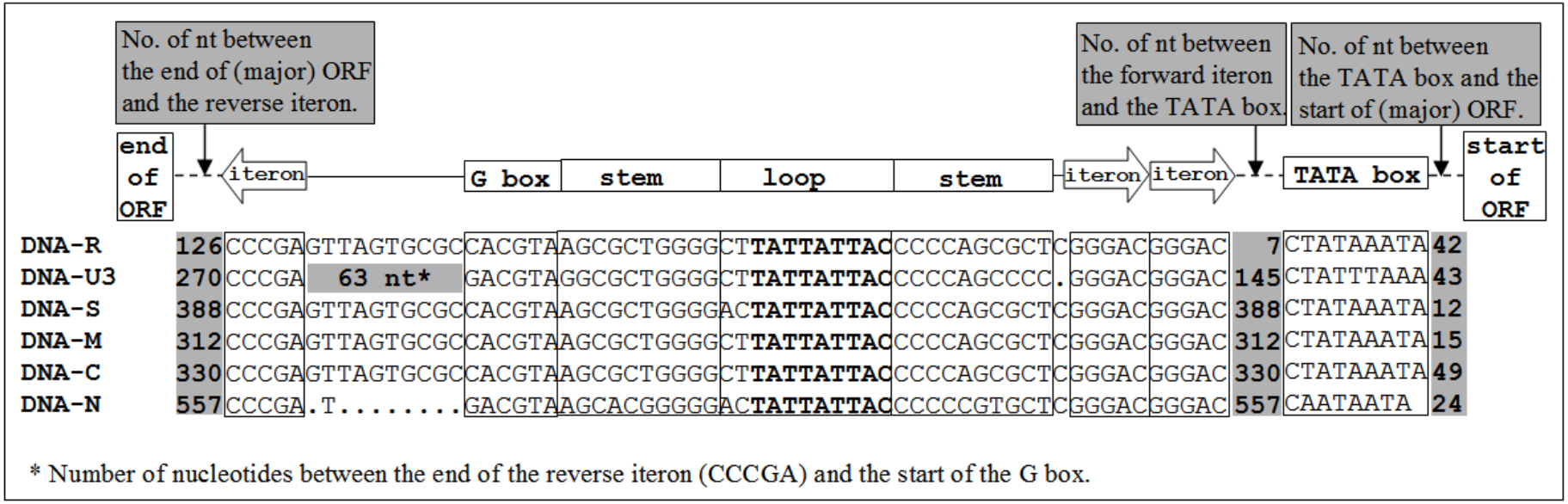



**Fig. 2-2a. Alignment of the intergenic region (CR-SL) of BBTV integal genome components.**

The iterons (in reverse and forward orientations), the loop sequence, G box and TATA box of BBTV integral genome components are aligned and compared. The dots within the sequences represent spaces in the alignment. The 9 nt conserved within the loop are in bold letters.

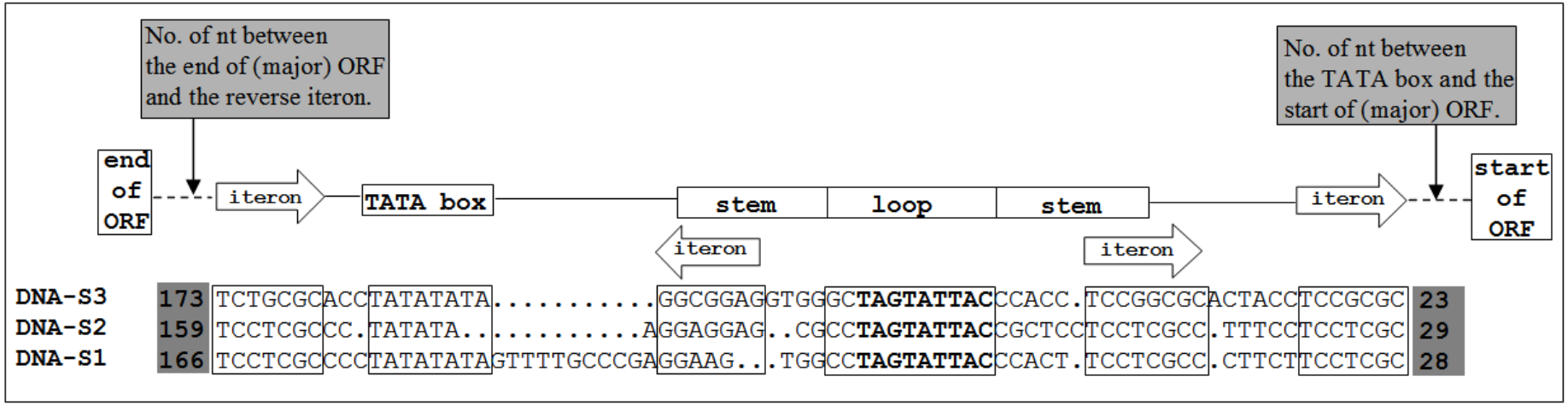



**Fig. 2-2b. Alignment of the intergenic regions of BBTV satellite DNAs.**

The conserved iterons (in forward and reverse orientations), TATA box, the stems and loop of the DNA-S1, -S2 and -S3 are compared. The dots within the sequences represent spaces in the alignment. The conserved 9 nt sequences within the loop are in bold letters. The figure is modified from Horser (2000).

region which is 93% conserved among the BBTV integral genome components. Furthermore, an almost complete direct repeat (ATAACAA[CG]AC[AG]CTATA TGA) can be found near the 5' end of the CR-M in BBTV DNA-U3, -S, -M, -C and -N, although not in DNA-R. At the start of viral DNA replication, short DNA primers are thought to anneal to the CR-M to initiate synthesis of second strand viral DNAs (Hafner *et al.,* 1997a).

The TATA box of BBTV integral genome components is located upstream of the translational start codon (ATG). The TATA box sequence of DNA-R, -S, -M, -C and -N is CTATAATA, while that of DNA-U3 is CAATAATTA (Beetham *et al.,* 1997). Interestingly, the CTATAATA sequence can also be found in the ORF of DNA-U3 (Beetham *et al.,* 1997).

Three transcription termination elements can also be found in the six BBTV integral genome components; (1) a polyadenylation (polyA) signal (AATAAA), (2) the G-T rich region containing a TGG sequence and (3) a consensus sequence [CAT]TGTAA (Beetham *et al.,* 1997). The polyA signal and the consensus [CAT]TGTAA are located either at the 3' end of the ORF or immediately downstream of the translational stop codon. However, the G-T rich region is always located downstream of the translational stop codon. These elements define the site of polyA and regulate the processing efficiency of transcripts. The consensus sequence T[AT]TGTA has been reported in the ORFs of other plant DNA viruses (Beetham *et al.,* 1997). The relative position of the CR-SL, iterons F1, F2 and R, G box, TATA box, CR-M, polyA signal and the ORF, are illustrated in Fig. 2-3a.

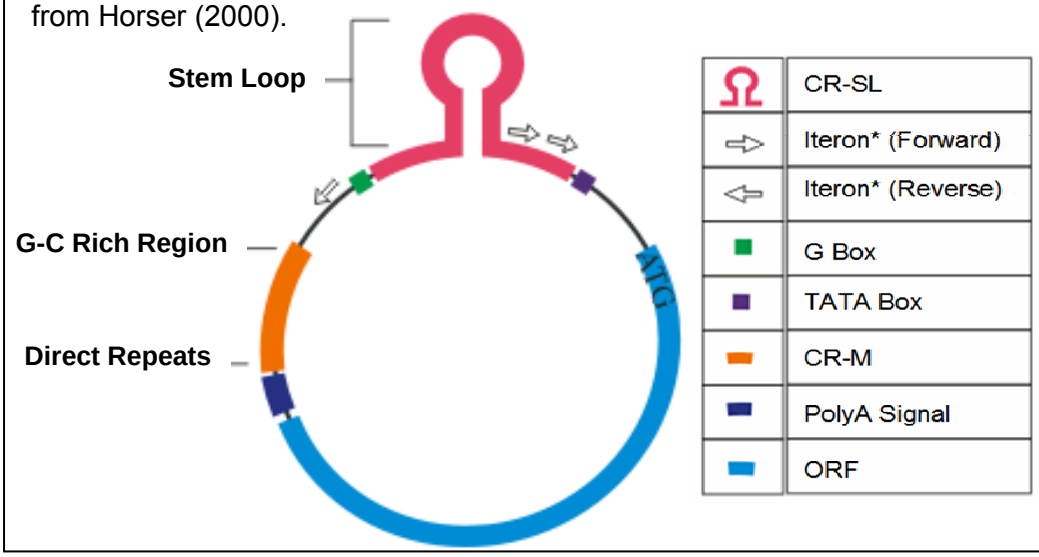
### *2.1.7. BBTV satellite DNAs*

Other cssDNAs, known as BBTV DNA-S1, -S2, -S3, -Y, -W1 and -W, are occasionally found associated with BBTV isolates (Horser *et al.,* 2001b; Bell *et al.,* 2002; Yeh *et al.,* 1994; Wu *et al.,* 1994). These DNA components are not found consistently with all isolates of BBTV, therefore, they are generally believed to be satellite DNAs of BBTV. DNA-S1, -S2, -Y, -W1 and -W2 were originally found with the Taiwanese isolates of BBTV (Horser *et al.,* 2001b; Yeh *et al.,* 1994; Wu *et al.,* 1994), while DNA-S3 was originally found with a Vietnamese isolate of BBTV (Bell *et al.,* 2002). The sequences of DNA-S2 and -W2 are highly homologous so the two may be considered to be the same component; the sequences of DNA-Y and -W1 are also essentially the same (Horser *et al.,* 2001b).

Both BBTV satellite DNAs and DNA-R encode the Rep in the virion-sense, although BBTV satellite DNAs do not contain the internal U5 ORF. An artificial replicative clone (1.1mer) of DNA-S1 has been shown to suppress DNA-R 1.1mer replication in transgenic banana cells, suggesting that BBTV DNA satellites and DNA-R may compete for resources in plant cells (Horser *et al.,* 2001a).

BBTV satellite DNAs and six BBTV integral genome components have several similar features. Firstly, each of the components is approximately 1 kb in size. Secondly, the TATA box is conserved. Thirdly, the transcription termination elements (i.e. the polyA signal, the G-T rich region and the [CAT]TGTAA consensus) are conserved. Fourthly, the stem-loop structure is conserved. Lastly, the loop (within the stem-loop structure) has the conserved TAnTATTAC consensus sequence at the apex (Horser *et al.,* 2001b; Bell *et al.,* 2000).

**a) BBTV integral genome includes DNA-R, -U3, -S, -M, -C and -N.**
Each of the DNA components of BBTV integral genome contains only one ORF, except BBTV DNA-R, which contains two ORFs (one internal to the other). The direct repeats are not found in DNA-R. The polyadenylation signals are within the ORF of DNA-R and –N; but are immediately downstream of the ORFs of DNA-U3, -S, -M and -C. The figure is modified from Horser (2000).




**b) Satellite DNAs of BBTV.**
In BBTV satellite DNAs, the stem-loop structure and the TAnTATTAC sequence at the loop apex are conserved; however, the rest of the CR-SL is not conserved. The CR-M sequence was found only in DNA-S1, but not in the other BBTV satellite DNAs.

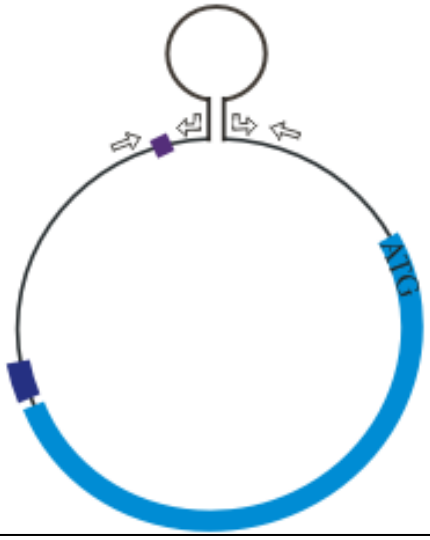
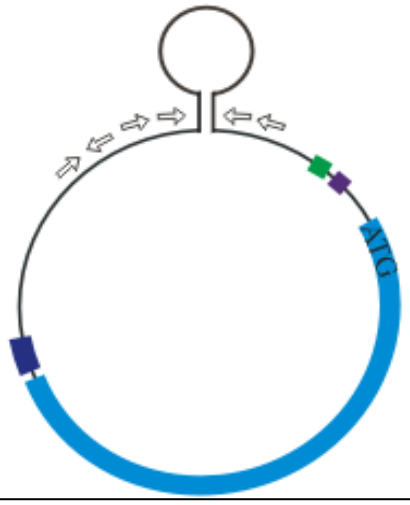


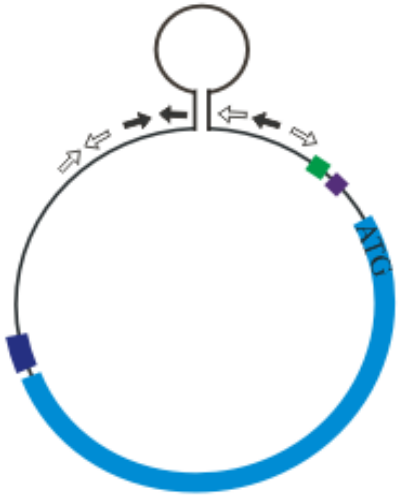

**Fig. 2-3. Genome organisation of nanoviruses**

These figures are schematic illustration of the general organisation of cssDNA genome components of nanoviruses and satellite DNAs of nanoviruses. The coloured boxes in the figures are used to symbolise regions with highly conserved sequences in the genome. Theses figures are not to scale.

**c) Integral genome of FBNYV and MDV.**
Genome organisation and iteron sequences of FBNYV and MDV are strikingly similar.




**d) Integral genome of SCSV.**
In SCSV genome, two different iteron sequences are conserved and are indicated by arrows of different shades in this figure.




**e) Satellite DNAs of FBNYV, MDV and SCSV.**
Genome organisation of satellite DNAs of BBTV, FBNYV, MDV and SCSV are strikingly similar.

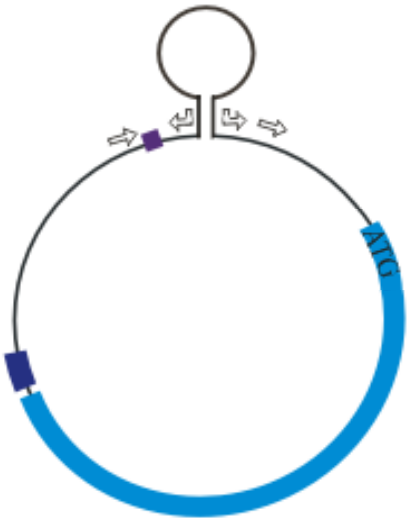



**Fig. 2-3. Genome organisation of nanoviruses**

The DNA satellites are generally believed to be encapsidated, moved and transmitted with the BBTV integral genome.

BBTV satellite DNAs have some features that are different from the BBTV integral genome components: (1) although the stem-loop structure and 9 nt consensus at the loop apex are both conserved in the satellite DNAs, the rest of the CR-SL sequence is different; (2) the satellite DNAs (except DNA-S1) do not has a conserved CR-M, (3) the TATA box is located immediately downstream of the stem-loop structure in the BBTV integral genome components, but the TATA box is located immediately upstream of the stem-loop structure in satellite DNAs (Horser *et al.*, 2001b; Bell *et al.*, 2002). The sequences of the intergenic regions and the genome organisation of the BBTV satellite DNAs are illustrated in Fig. 2-2b and 2-3b, respectively.

To investigate the prevalence of BBTV satellite DNAs, BBTV-infected banana samples from various geographical regions were tested by Southern analysis, using the ORF sequence of DNA-S1 as the probe (Horser *et al.*, 2001b). The S1 probes could detect similar sequences such as the ORF of DNA-S2, but not the less homologous Rep ORF of DNA-R (Horser *et al.*, 2001b). Hybridisation signals were observed in isolates from Tonga (1/1), Samoa (1/2), the Philippines (2/3), Taiwan (1/1) and Vietnam (13/13). However, hybridisation signals in the Samoan and several of the Vietnamese isolates were weak, indicating that the detected sequences were less homologous to DNA-S1. No hybridisation was detected in Australian (0/13), Egyptian (0/2), Fijian (0/1) or Indian (0/1) isolates. The nucleotide sequences of DNA-R from the tested isolates shared > 83.5% identity with DNA-R of the Australian isolate (Bell *et al.*, 2002).

There may be satellite DNAs, however, with sequences distinct from DNA-S1 and -S2, that were not detected using the S1 probe.

In an extended study, BBTV-infected banana samples from Vietnam were tested for satellite DNAs, using the ORF sequence of the Vietnamese BBTV DNA-S3 as a probe for Southern analysis (Bell *et al.,* 2002). Hybridisation signals were observed in 51 % (41/81) of the samples, that were collected from various regions across Vietnam (Bell *et al.,* 2002). The 41 positive samples were then tested with a pair of PCR primers that anneal to the ORF of DNA-S3. PCR amplification only occurred in 32 of the 41 samples, indicating sequence variation among the satellite DNAs. There may be satellite DNAs that were not detected by the S3 probe and primers under the experimental conditions.

### *2.1.8. Rolling circle replication*

The proposed model for BBTV replication (Fig.2-4) has been based on the rolling circle replication (RCR) of geminiviruses (Gronenborn, 2004; Hafner, 1998; Laufs *et al.,* 1995a). After entering the cell of the host plant, the virus is uncoated to free the viral cssDNAs which enter the cell nuclei. DNA primers then anneal to the CR-M within the intergenic region of the cssDNAs to initiate synthesis of the second strand (also known as the negative sense or complementary strand) of viral DNAs (Hafner *et al.,* 1997a). The synthesis of viral DNA is aided by host DNA polymerases. Transcriptionally active dsDNAs then express the viral proteins including the Rep. To switch plant cells to S-phase for optimal viral replication, the Clink protein encoded by DNA-C presumably would also be expressed at this stage (Aronson *et al.,* 2000).

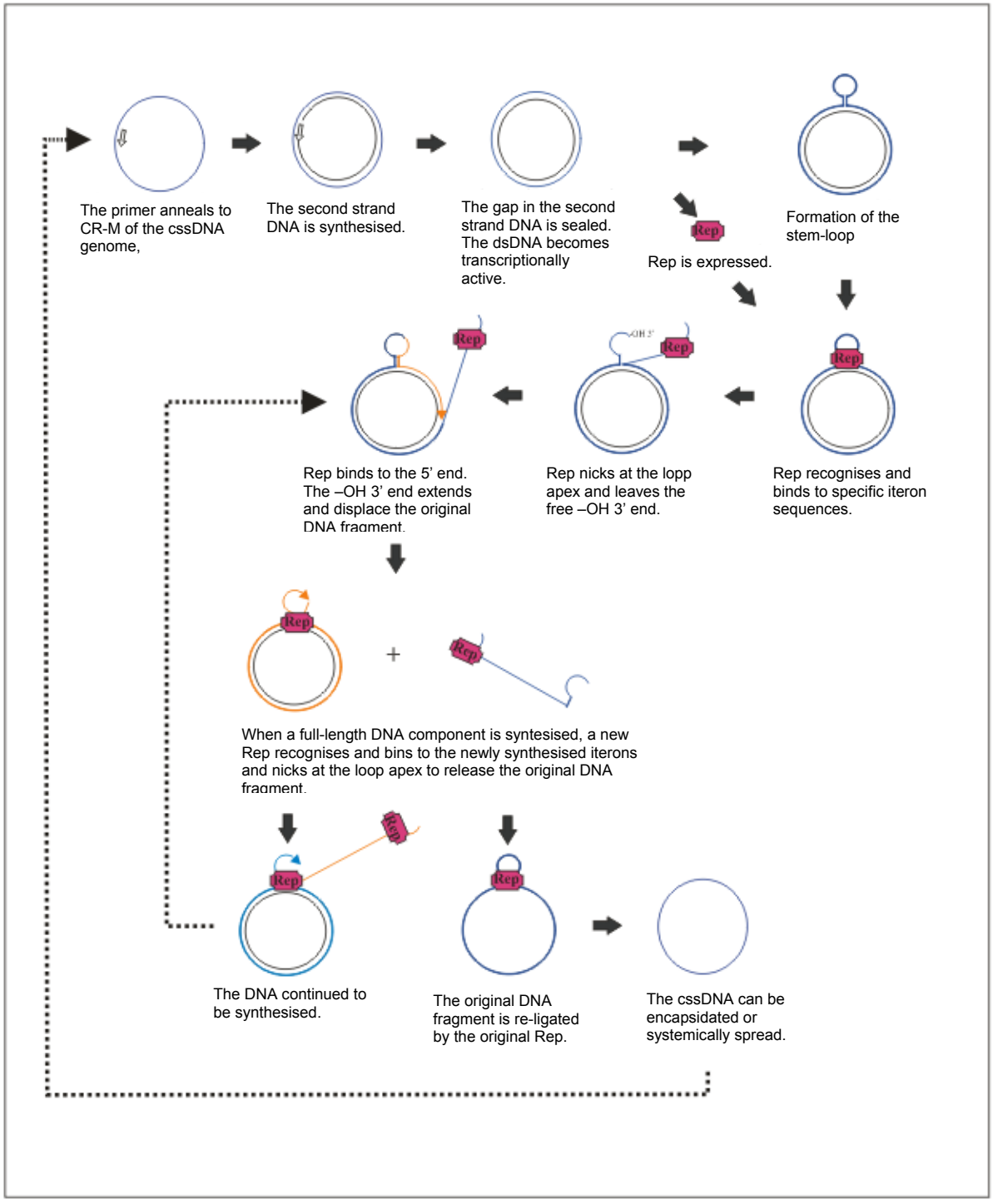



**Fig. 2-4. The proposed model for rolling circle replication**

This schematic illustration is modified from Hafner (1998).

The Rep recognises and binds to specific iterons anchoring the stem-loop structure of the viral cssDNA and nicks the positive strand at the 9 nt conserved sequence (TAnTATT↓AC[9]) at the loop apex. The nicking creates a free 3'-OH group, that initiates synthesis of positive sense ssDNA to displace the free 5' strand. Once a full-length viral ssDNA component is synthesised, a new Rep cleaves the newly synthesised stem-loop at the TAnTATT↓AC, to allow a new round of synthesis. The original Rep, which is still bound to the 5' end of the released ssDNA monomeric unit, ligates the DNA molecule back into the circular conformation, ready for further transcription and replication.

### *2.1.9. The "master" Rep*

DNA-R is presumably the only BBTV genome component that is essential for replication of the BBTV integral genome. The Rep encoded by BBTV DNA-R is believed to be the "master" Rep (M-Rep), because it not only initiates replication of itself, but also replication of the other five cssDNAs in the BBTV integral genome (Horser *et al.,* 2001a). BBTV satellite DNAs also encode a Rep, but can only initiate self-replication, not replication of the BBTV integral genome (Horser *et al.,* 2001a).

Recognition of specific iterons is executed by approximately the first 30 amino acid (aa) residues of the Rep, that comprises the rolling circle replication motif 1 (RCR-1) (Timchenko *et al.,* 2000; Arguella-Astorga and Ruiz-Medrano, 2001). Rep proteins with similar RCR-1 can often replace each other for initiating viral replication. For example, the M-Rep encoded by either MDV, SCSV or FBNYV can bind to similar iterons in the integral genome components of all three

9 The "↓" represents site of nicking.

viruses to initiate genome replication (Fig. 2-3c and 2-3d) (Timchenko *et al.,* 2000). The more similar the iterons are, the more efficient replication can be (Timchenko *et al.,* 2000). BBTV M-Rep is unlikely to initiate replication of the other nanoviruses because the sequence of the first 30 aa of BBTV M-Rep is distinct from that of other nanoviruses. Nevertheless, the BBTV integral genome components and the begomoviruses (family *Geminiviridae*) have similar iterons (Horser, 2000), suggesting that BBTV M-Rep may initiate replication of begomoviruses, and *vice versa*.

Furthermore, the satellite DNAs of nanovirues have similar iterons (Fig. 2-3b and e), therefore, replication of all satellite DNAs may be initiated by any of their encoded Reps. The mastreviruses (family *Geminiviridae*) and satellite DNAs of nanoviruses also have similar iterons, suggesting that the Rep encoded by satellite DNAs of nanoviruses may initiate replication of mastrevirus, and *vice versa* (Horser, 2000).

### *2.1.10. Rep is a multi-functional protein*

Alignment of aa sequences has revealed several functional domains in the Rep of BBTV, including an RCR domain, an ATPase domain and possibly an oligomerisation domain (Fig. 2-5a).

The RCR domain, that is located near the N-terminus of the BBTV Rep, consists of the RCR motifs 1, 2 and 3 (RCR-1, -2 and -3). This domain is responsible for site-specific interaction between the Rep protein and the viral DNA (Jupin *et al.,* 1995; Orozco *et al.,* 1997). RCR-1, -2 and -3 are conserved among RCR proteins encoded by the genomes of nanoviruses and geminiviruses,

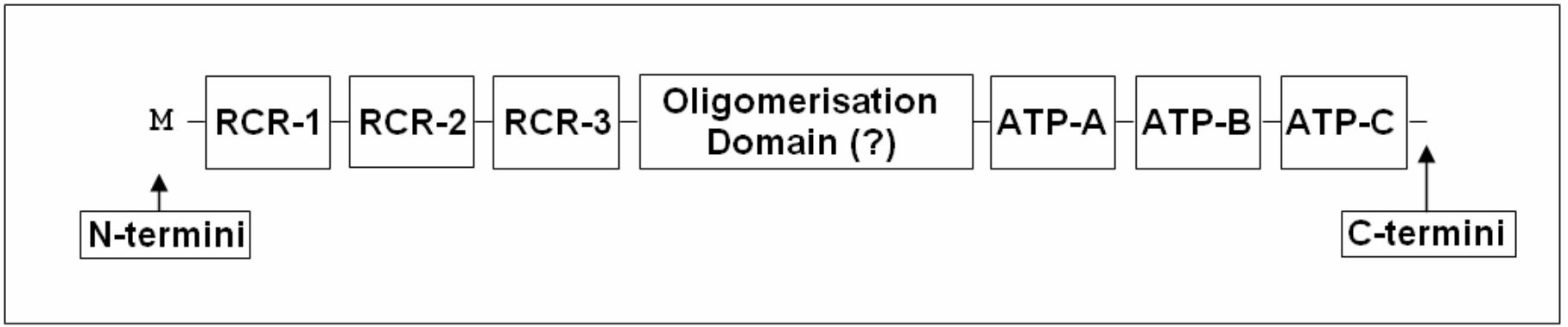



**Fig. 2-5a. Schematic representation of the conserved regions in the Rep encoded by BBTV DNA-R and BBTV satellite DNAs.**

The open boxes represent conserved domains or motifs. RCR = Rolling circle replication; ATP = ATPase. This figure is not to scale.

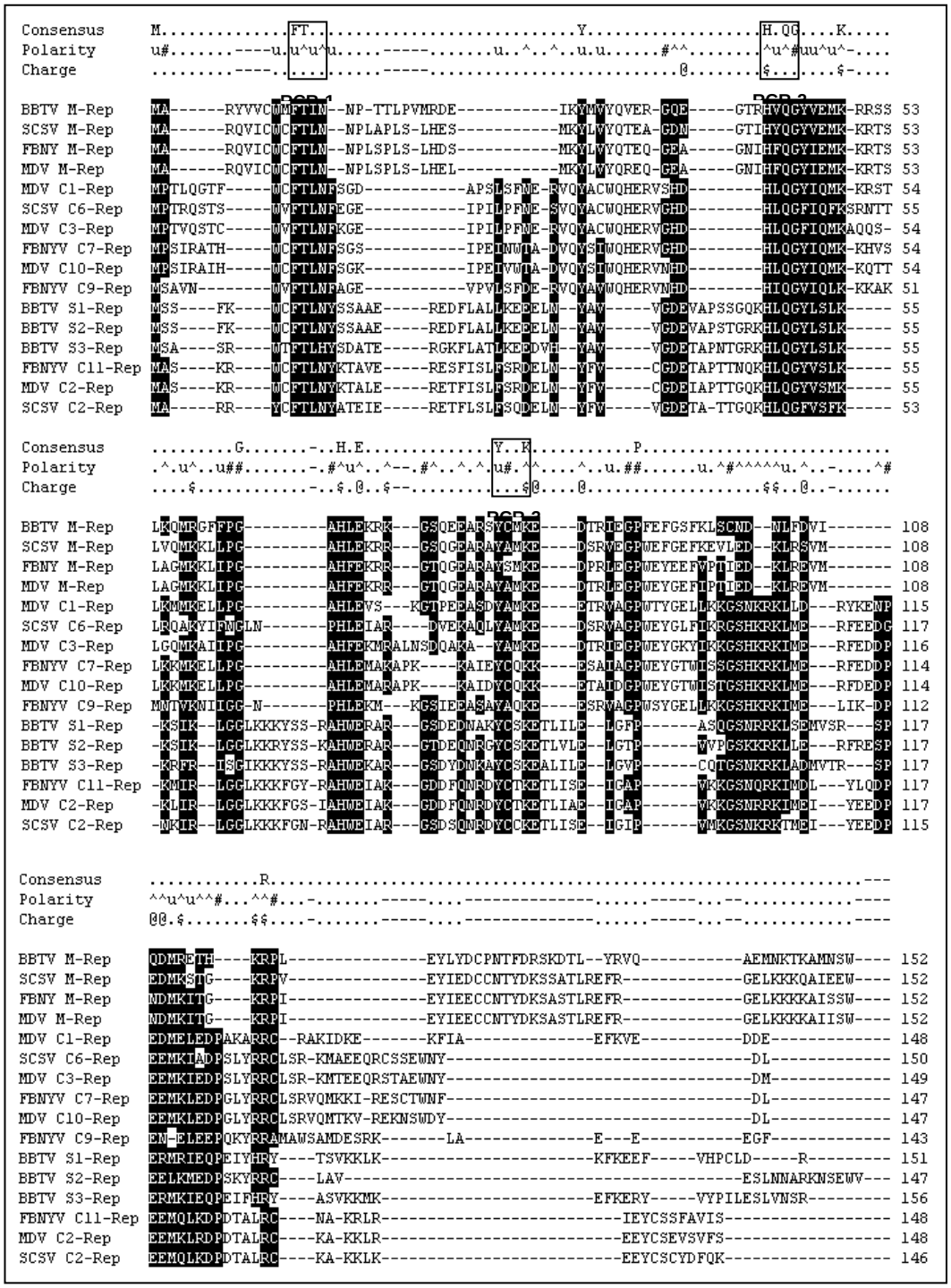


**Fig. 2-5b. Alignment of Rep encoded by the DNA-R and the satellite DNAs of nanoviruses** (continued to next page)

The “Consensus” pattern shows amino acid residues that are conserved in all sequences. The “Polarity” pattern shows the polarity conserved in ≥ 75 % of the sequences: “u” indicates hydrophobic residues (FILMVWY); “#” indicates neutral residues (PCAG); and “^” indicates hydrophilic/polar residues (TSKQNHEDR).

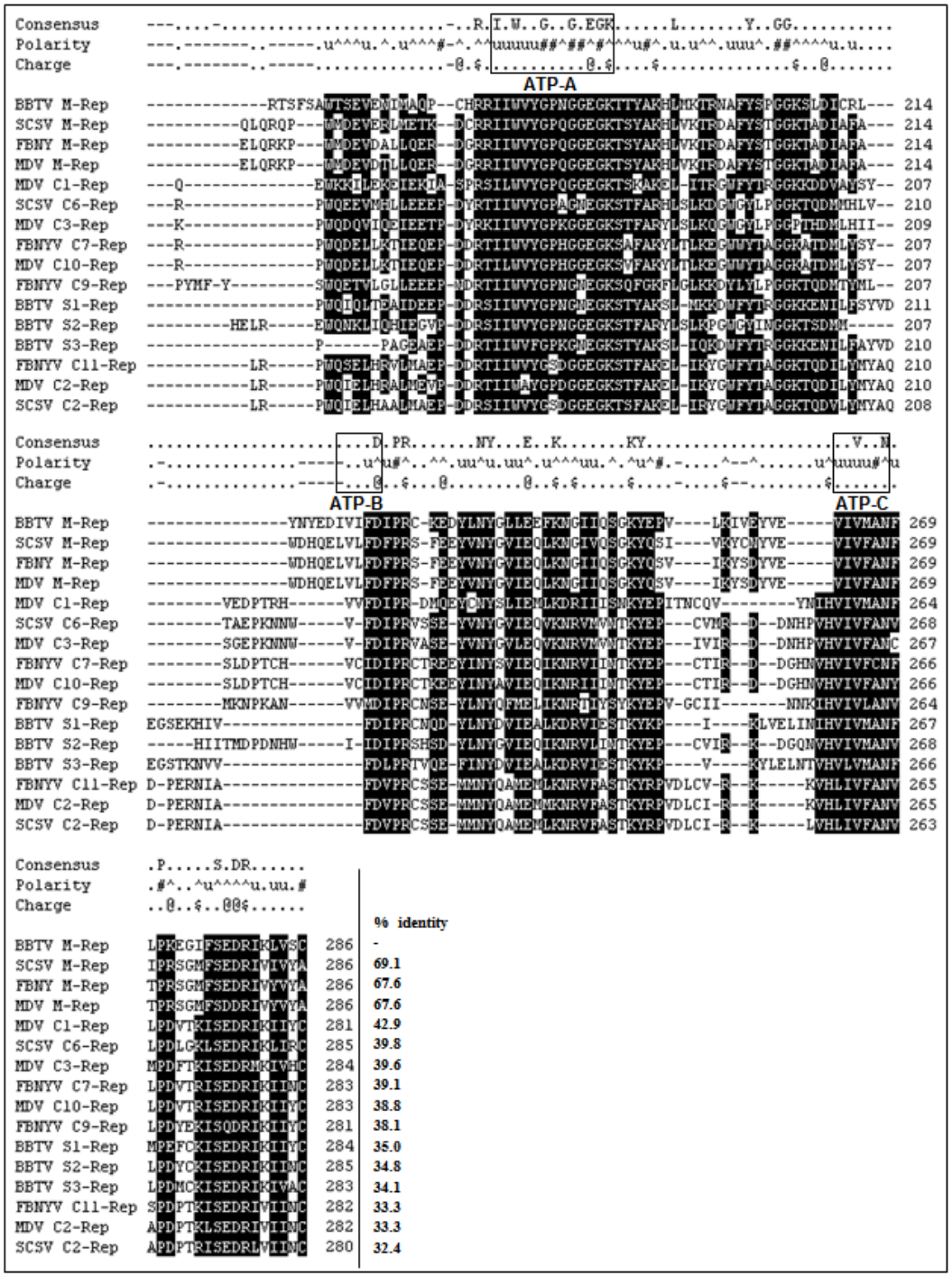



**Fig. 2-5b. Alignment of Rep encoded by the DNA-R and the satellite DNAs of nanoviruses** (continued from previous page)

The "Charged" pattern shows the charged residues conserved in ≥ 75 % of the sequences: "@" indicates acidic/negatively charged residues (ED); and "$" indicates basic/positively charged residues (KHR). The residues which match the "Polarity" pattern exactly were shadowed by black. The putative functional motifs, which were identified in reference to studies of Koonin (1993), are boxed. The percentages of identity to BBTV M-Rep are listed at the end of each sequence.

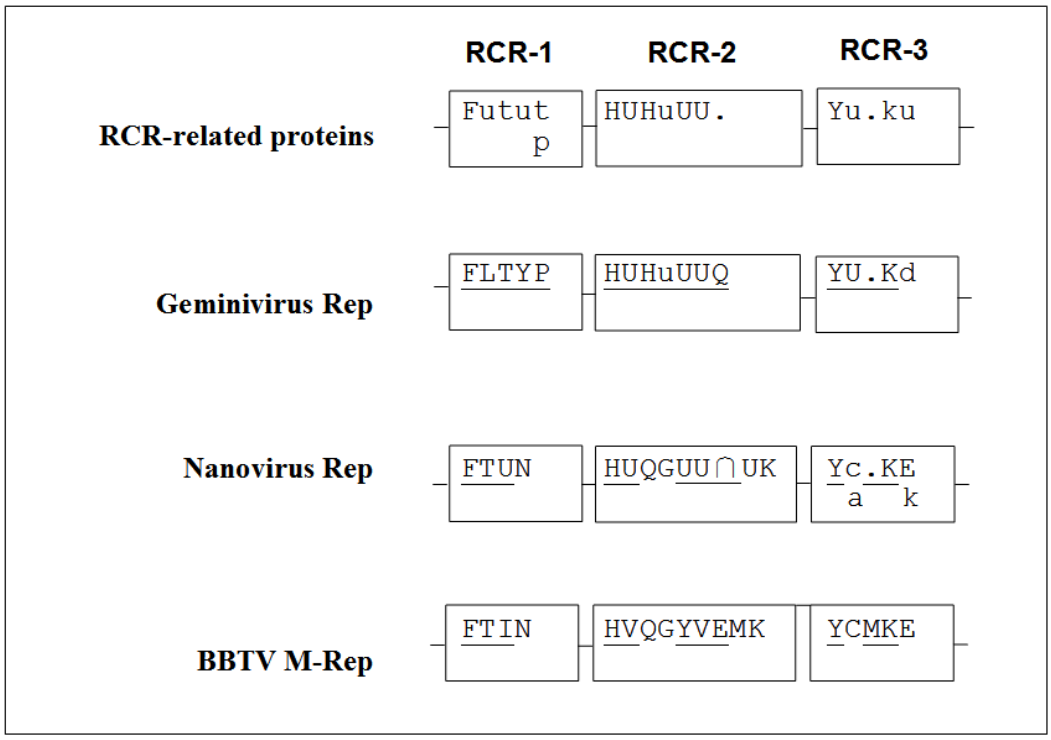



**Fig. 2-5c. Comparison of motifs 1, 2 and 3 of the RCR domains**

The typical consensus sequences for RCR-related proteins and the consensus sequences of geminivirus Rep are from the work of Ilyina and Koonin (1992). The consensus sequences for the Rep of nanoviruses and nanovirus satellite DNAs are from the alignment of Fig. 2-5b. Upper cases indicate the amino acid residues that are conserved in ≥ 75 % of the aligned sequences. Lower cases indicate the amino acid residues that are conserved in ≥ 50 % (but < 75 %) of the aligned sequences. The underlined amino acid residues are conserved from the typical consensus sequences of RCR motifs.

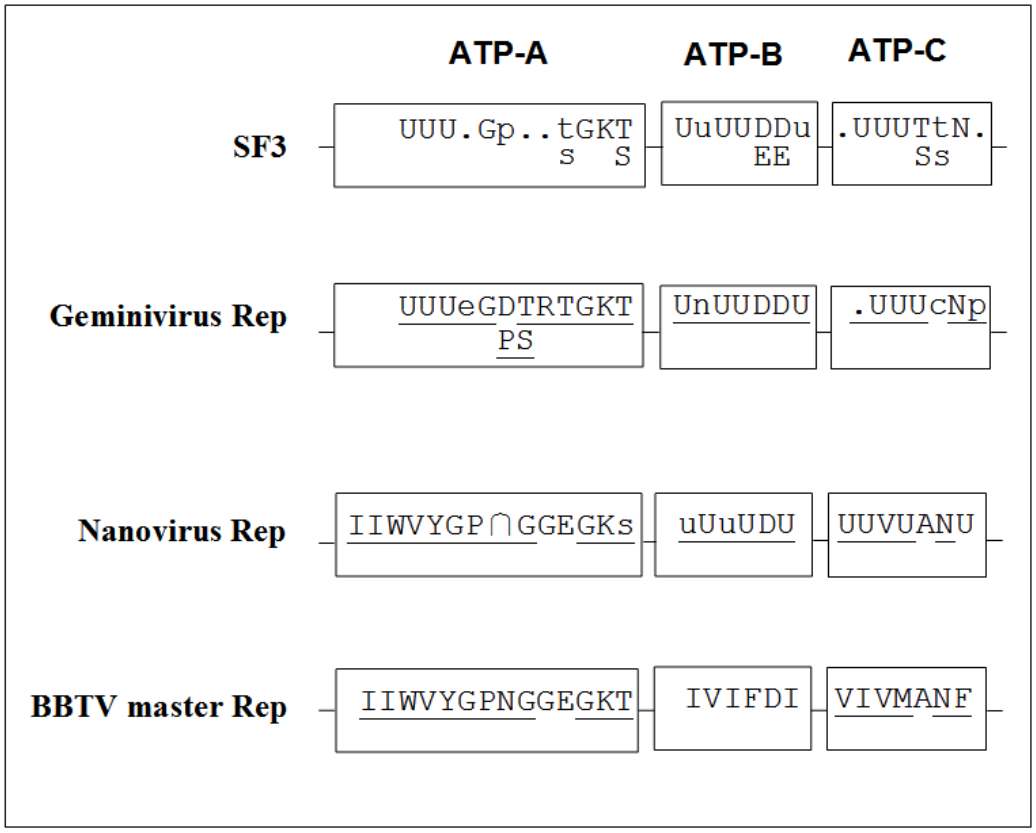



**Fig. 2-5d. Comparison of motifs A, B and C of the ATPase domains**

The typical consensus sequences for SF3 proteins and the consensus sequences of geminivirus Rep are from the work of Koonin (1993). The consensus sequences for the Rep of nanoviruses and nanovirus satellite DNAs are from the alignment of Fig. 2-5b. Upper cases indicate the amino acid residues that are conserved in ≥ 75 % of the aligned sequences. Lower cases indicate the amino acid residues that are conserved in ≥ 50 % (but < 75 %) of the aligned sequences. The underlined amino acid residues are conserved from the typical consensus sequences of ATP motifs.

as well as the plasmids of eukaryotes, bacteria, bacteriophages, cyanobacteria and archaebacteria (Ilyina and Koonin, 1992).

Motif RCR-1 contains the consensus sequence [FILV][ILV][ILVT]YP in the Rep of pMV158-related plasmids and geminiviruses (Fig. 2-5b and 2-5c) (Ilyina and Koonin, 1992). In the Rep of nanoviruses, the sequence of RCR-1 deviates from the above consensus, but the region still forms a β-sheet that can recognise and bind to specific iterons (Vega-Rocha *et al.*, 2007). The sequence FTIN can be found in the BBTV M-Rep and the sequence FTLN can be found in BBTV satellite DNAs (Fig. 2-5b and 2-5c). Removing the RCR-1 can inactivate the nicking and non-covalent binding activities of the Rep (Orozco *et al.*, 1997; Orozco and Hanley-Bowdoin, 1998).

Consensus sequences of motifs RCR-2 and -3 of various Rep proteins are aligned and compared in Fig. 2-5c. Motif RCR-2 has the consensus sequence H[FILYWVTS]H[ILVMCA][FILWYVM][FILWYVMCA] in the Rep of pMV158-related plasmids and geminiviruses (Ilyina and Koonin, 1992). The sequence deviates slightly in nanoviruses, but the general feature ∩U∩UUU[10] is conserved. RCR-2 in the Rep of BBTV DNA-R and BBTV satellite DNAs has the sequence H[V<u>L</u>]QGY[V<u>L</u>][11] (i.e. ∩U∩#UU) (Fig. 2-5b and 2-5c). The role of RCR-2 is most likely to be metal binding (Hastie, 2001). An ∩U∩UUU →AAAUUU mutation was shown to abolish the ability of Tomato golden mosaic virus (TGMV) Rep to initiate replication and to bind, nick and re-ligate DNA,

10 The symbol "∩" indicates hydrophilic/polar residues, including TSKQNHEDR. The "U" indicates hydrophobic residues, including FILWYVM. The "#" indicates neutral residues, including PCAG.

11 The underlined residues have been found only in the Rep of BBTV DNA satellites.

indicating the significance of Rep-metal ion interactions (Orozco and Hanley-Bowdoin, 1998).

Motif RCR-3 has the consensus sequence [ILVMATS]xxY[ILVMCA] x[KH] in the Rep of pMV158-related plasmids and geminiviruses (Ilyina and Koonin, 1992). This consensus sequence is fully conserved in the Rep of nanoviruses. RCR-3 of BBTV M-Rep has the sequence ARSYCMK; while the sequence [AKR][GAK]YCSK is found in Rep of BBTV DNA satellites. Studies of Rep encoded by the bacteriophage ΦX174 and Tomato yellow leaf curl virus (TYLCV) suggest the highly conserved Y residue forms covalent phosphotyrosine bonds with DNA (Laufs *et al.,* 1995b; van Mansfeld *et al.,* 1986). Mutation studies have shown that Y and K residues of Rep are critical for Rep to bind, nick and re-ligate the ssDNA genome and to initiate replication of geminiviruses and nanoviruses (Hoogstraten *et al.,* 1996; Orozco and Hanley-Bowdoin, 1998; Timchenko *et al.,* 1999).

An oligomerisation domain is thought to be present between the RCR and ATPase domains of BBTV Rep proteins. The Rep of geminiviruses has the oligomerisation domain that interacts with plant Rb-related proteins (pRBR), proliferative cell nuclear antigen (PCNA) and viral coat proteins, and may bind with other Rep proteins to form multimers (Orozco *et al.,* 2000; Arguello-Astorga *et al.,* 2004; Shepherd *et al.,* 2005; Bagewadi *et al.,* 2004; Malik *et al.,* 2005). The oligomerisation domain in the Rep of geminiviruses is also involved in non-specific ssDNA binding and appropriate assembly of Rep at the replication origin (Gomez-Llorente *et al.,* 2005; Hickman and Dyda, 2005). Oligomerisation of BBTV Rep or that of the other nanoviruses has not been thoroughly

investigated but the Rep of nanoviruses may not interact with pRBR because Rep proteins do not encode the typical Rb-binding (Clink) motif LxCxE (Gronenborn, 2004). The Clink motif is located in the Clink proteins encoded by DNA-C in nanoviruses (Aronson *et al.,* 2000; Wanitchakorn *et al.,* 2000a). Oligomerisation of the M-Rep, however, has been observed for nanoviruses (Vega-Arreguin *et al.,* 2005). The M-Rep of CFDV has also been shown to interact with itself (Merits *et al.*, 2000).

The ATPase domain consists of AAA+ ATPase[12] motifs A, B and C (ATP-A, -B and -C) and is located near the C-terminus of BBTV Rep (Gorbalenya *et al.,* 1989; Koonin, 1993). The motifs ATP-A, B and C are conserved in all the superfamily 3 (SF3) helicases, including the Rep of small DNA and RNA viruses (Gorbalenya *et al.,* 1989; Koonin, 1993; Clérot and Bernardi, 2006). The consensus sequences of ATP-A, -B and -C of various Rep proteins are compared in Fig. 2-5b and 2-5d. In these viral Rep proteins, the ATPase domain may contribute to the melting of the replication origin and unwinding of replicative intermediates. Truncated Rep proteins without the ATPase domain (T-Rep) lose ATPase activity, but maintain RCR and oligomerisation activities (Hong and Stanley, 1995; Noris *et al.,* 1996; Brunetti *et al.,* 2001).

Motif ATP-A has the consensus sequence [FILWVMC] [FILWYVCASQE][FILVM][IVYMPCTSKQHER]G[IPASKQED][IVPAGTSQR] [GTSNHDR][VCAGTSD]GK[TSN] in most SF3 helicases (Koonin, 1993). ATP-A of BBTV Rep has the sequence IIWV[YF]GP[NK]G[GN]EGK[TS]. The

12 AAA+ ATPase is the abbreviated name for the ATPase associated with various cellular activities.

consensus sequence is slightly divergent in Rep of nanoviruses, but the general $U_nGx_nGK[TS]$ feature is conserved. The positively charged K is a fully conserved residue and binds to the terminal phosphate group of a nucleoside-triphosphate (NTP), which forms a complex with a $Mg^{2+}$ ion (Schlee *et al.,* 2001). A K→A mutation abolishes helicase and ATPase activities of the protein, perhaps by disturbing its quaternary structure (Pause and Sonenberg, 1992; Parsell *et al.,* 1994).

Motif ATP-B of most SF3 helicases has the consensus sequence [FILYVASR][FILVCAN][ILWYVMCTHR][FILWYVMH][LKNED][ED] (Koonin, 1993). The sequences of motif ATP-B are conserved in the Rep of nanoviruses, but usually with only one D residue at the end. Motif ATP-B of BBTV Rep has the sequence [LIV][VI][IV][FI]D. The D residue is negatively charged and chelates the $Mg^{2+}$ ion of the $Mg^{2+}$-NTP complex (Gorbalenya *et al.,* 1989). The mutation of D to uncharged residues interferes with both helicase and ATPase activities (Gorbalenya *et al.,* 1989). Approximately 25 αα downstream of the D of motif ATP-B, motif ATP-B', characterised by a consensus sequence KU, is often found (Koonin, 1993). Motif ATP-B' is thought to be responsible for non-specific ssDNA binding required for coupling helicase and ATPase activities (Yoon-Roberts *et al.,* 2004; Pause and Sonenberg, 1992). The Rep with a K→A mutation in motif B' loses helicase activity, but maintains basal ATPase activities (Yoon-Roberts *et al.,* 2004; Pause and Sonenberg, 1992).

Motif ATP-C is sometimes known as the “sensor 1” motif (Neuwald *et al.,* 1999). The motif ATP-C of most SF3 helicases has the consensus sequence [FILVMCAGTS][FILWVT][FILYVMCAS][FLYVMCTS][IVMCAGTSN]N (Koonin, 1993). Motif ATP-C is conserved in Rep of nanoviruses as a stretch of hydrophobic residues followed by XN. The fully conserved N residue interacts with a water molecule that is probably the nucleophile for ATP hydrolysis (Lenzen *et al.,* 1998). An N→A mutation has been shown to almost eliminate the ATP hydrolysis function of ATPase without disturbing the NTP-binding ability of the protein (Hattendorf and Lindquist, 2002).

# 2.2. Control

# 2.2. Control

BBTV is spread either by the banana aphid (*Pentalonia nigronervosa*) (Magnaye and Valmayor, 1995) or via vegetative propagation of infected plant propagules (Wardlaw, 1961). Control of BBTV in Australia depends on a four-pronged approach which includes registration of plantations, eradication of diseased plants, controlled replanting with known virus-free stock and domestic quarantine zones (Allen, 1978a,b; Dale, 1987). Importantly, all control methods are supported by legislation.

An alternative approach for controlling spread of BBTV is to generate BBTV-resistant banana plants. Unfortunately, BBTV resistance cannot be introduced into edible banana varieties by conventional breeding because (1) there is no member of the genus *Musa* that is completely immune to BBTV and (2) edible banana varieties are sterile (Dale, 1987).

Genetic engineering offers new opportunities for generating BBTV-resistant banana. Transforming plants with part of the viral genome may generate plants resistant to the virus from which the transgene sequence was derived (Sanford and Johnston, 1985). This concept is known as pathogen-derived resistance (PDR), and has been applied successfully in various plant species to confer resistance against many RNA viruses (Goldbach *et al.,* 2003). The use of PDR to control DNA viruses has been far less successful and has concentrated almost exclusively on geminiviruses (Vanderschuren *et al.,* 2007;

Hanley-Bowdoin *et al.,* 2004a,b; Goldbach *et al.,* 2003). Since both nanoviruses and geminiviruses are cssDNA viruses that infect plants, it is feasible that PDR strategies to control geminiviruses may also be successful against nanoviruses such as BBTV.

The PDR mechanism may involve interference of viral protein activities or viral gene expression. Full-length or truncated, wild-type or mutated viral sequences in sense, anti-sense or as inverted repeats, have all been used successfully to generate PDR (Goldbach *et al.,* 2003). For geminiviruses, genes that encode the Rep, CP, movement protein (MP), nuclear shuttle protein (NSP) and the replication enhancer protein (REn) have all been used in attempts to generate PDR against geminiviruses (Vanderschuren *et al.,* 2007; Hanley-Bowdoin *et al.,* 2004a, b). Non-coding regions of geminiviruses have also been used to generate PDR against geminiviruses (Yang *et al.,* 2004). All of the above PDR strategies are discussed below, except the use of REn genes because these genes have not been found in nanoviruses.

### *2.2.1. Resistance mediated by Rep-encoding genes*

Rep proteins of nanoviruses and geminiviruses are multi-functional proteins responsible for initiating replication of the viral cssDNA genome. Most of the studies on Rep-mediated resistance have focused on geminiviruses. Plants transformed with full-length or partial geminiviruses Rep genes often show resistance against the virus which the Rep genes have been derived from. Two patents have been granted for all PDR strategies against geminiviruses that are mediated by Rep genes, including the full-length or partial, wild-type or mutated Rep genes in sense or anti-sense orientation or as inverted repeats (Hanley-Bowdoin *et al.,* 2004a; Polston *et al.,* 2005).

**Protein-mediated mechanism**

Rep transgenes may confer resistance against geminiviruses by interfering at several stages during viral replication. In the model proposed for geminiviruses, after the virus enters host cells, the viral genome is uncoated to synthesise the second strand (negative sense) viral DNA. The double-stranded viral DNA would express viral proteins such as Rep. Initial interference with viral replication may occur during this stage of Rep transcription because Rep is sometimes the suppressor of its own expression (Sunter *et al.,* 1993; Hong and Stanly, 1995; Brunetti *et al.,* 2001; Hanley-Bowdoin *et al.,* 2004a). For example, *Nicotiana benthamiana* plants that were transformed with a truncated Rep (T-Rep) gene from *Tomato yellow mosaic leaf curl Sardinia virus* (TYLCSV) showed strain-specific resistance against TYLCSV (Brunetti *et al.,* 1997; 2001; Lucioli *et al.,* 2003). This T-Rep gene did not possess the C-terminal ATPase domain and was unable to initiate viral replication but still retained the ability to bind specifically to native Rep promoter (Brunetti *et al.,* 1997; 2001; Lucioli *et al.,* 2003). As such, the T-Rep can bind to the viral Rep promoter and strongly suppress, although not completely abolish, transcription of wild-type Rep gene (Brunetti *et al.,* 2001; Lucioli *et al.,* 2003). The limited amount of wild-type Rep that was expressed from the suppressed viral Rep promoter then competed with the T-Rep for substrates such as DNA binding sites (Brunetti *et al.,* 2001; Lucioli *et al.,* 2003). Due to the fact that Rep usually binds to DNA as a monomer and then forms double hexamers, wild-type Rep would form defective double hexamers with T-Rep and fail to initiate replication (Brunetti *et al.,* 2001; Lucioli *et al.,* 2003).

In addition to TYLCSV, T-Rep proteins of TYLCV and TGMV have also

been used to confer strain-specific resistance against infection of TYLCV and TGMV, respectively (Noris *et al.,* 1996; Antignus *et al.,* 2004; Hanley-Bowdoin *et al.,* 2004a). Evidence of protein-mediated resistance, such as high mRNA levels of the T-Rep or suppression of the native Rep promoters, has been observed in resistant plants (Brunetti *et al.,* 1997; Antignus *et al.,* 2004; Hanley-Bowdoin *et al.,* 2004a). T-Rep proteins of *Tomato leaf curl New Delhi virus* (ToLCNDV) have also been shown to suppress replication of ToLCNDV in transiently transformed *N. tabacum* (tobacco) protoplasts as well as in *N. benthamiana* plants. Transient expression of T-Rep proteins were confirmed by immunoblots, suggesting suppression of replication also occurred at the protein level (Chatterji *et al.,* 2001).

Full-length Rep mutants (with defective ATPase domain) have also been shown to suppress replication of geminiviruses. For example, Rep mutants of *Tomato mottle virus* (ToMoV), *Bean golden mosaic virus* (BGMV) and *Bean golden yellow mosaic virus* (BGYMV) suppressed replication of ToMoV, BGMV or BGYMV, respectively (Stout *et al.,* 1997; Hanson and Maxwell, 1999). The interference mechanism is presumably similar to that of T-Rep described above (Hanley-Bowdoin *et al.,* 2004a).

T-Rep can sometimes confer broad-spectrum resistance against heterologous geminiviruses. For example, the T-Rep of TYLCSV has been used to confer resistance against TYLCV (Lucioli *et al.,* 2003). Further, the T-Rep of ToLCNDV suppressed replication of *African cassava mosaic virus* (ACMV), *Huasteco yellow vein virus* (HYVV), and *Potato yellow mosaic virus* (PYMV) (Chatterji *et al.,* 2001). The mechanism for resistance against heterologous viruses was different

from resistance against homologous viruses (Lucioli *et al.,* 2003; Chatterji *et al.,* 2001). The proposed mechanism was that T-Rep and the heterologous wild-type Rep formed hetero-oligomers (presumably double hexamers) that could not recognise specific binding sites on the viral DNA genome (Lucioli *et al.,* 2003). The hetero-oligomers could not recognise and bind to the native Rep promoter to suppress transcription. Because the hetero-oligomers could not recognise and bind to the iterons to initiate genome replication, genome replication was possibly suppressed.

The oligomerisation domain of Rep appears to be essential for protein-mediated resistance against heterologous virus (Luciolli *et al.,* 2003; Chatterji *et al.,* 2001). Rep can still bind however, to native Rep promoter as a monomer (Hanley-Bowdoin *et al.,* 2004a). For example, in a study of TGMV, a full-length Rep with a defective oligomerisation domain was able to bind to native Rep promoter and strongly suppress transcription of Rep (Hanley-Bowdoin *et al.,* 2004a).

Motif RCR-3 of Rep is responsible for covalent binding between Rep and viral DNA (Laufs *et al.,* 1995b; van Mansfeld *et al.,* 1986). A full-length Rep mutant (with defective RCR-3) of *Maize streak virus* (MSV) was found to suppress replication of MSV in embryogenic calli of *Digitaria sanguinalis* (Shepherd *et al.,* 2007). Calli failed to regenerate into plants however, probably because the Rb-binding motif of Rep interfered with the cell cycle. Calli that were transformed with a full-length Rep mutant with both a defective RCR-3 and Rb-binding motif were able to be regenerated into plants that were resistant to MSV (Shepherd *et al.,* 2007). It was postulated that the Rep mutant with defective

RCR-3 may have suppressed replication of viruses by non-covalent binding to the native Rep promoter to suppress transcription (Shepherd *et al.,* 2007). The mutant and wild-type Rep might also assemble defective oligomers that could not nick or re-ligate the viral genome to initiate viral replication (Shepherd *et al.,* 2007).

Protein-mediated resistance mediated by Rep is almost always the result of a defective protein because, while wild-type Rep proteins are able to suppress Rep transcription, wild-type Rep proteins are also functional and can act in place of suppressed native Rep to support viral replication.

Protein-mediated resistance against geminiviruses is often unstable and cannot tolerate high levels of viral inoculation. Transforming plants with defective Rep often only attenuates or delays disease symptoms caused by geminiviruses, and rarely confers complete immunity. This is because the defective Rep protein needs to be highly expressed in order to confer PDR by a protein-mediated mechanism. Furthermore, some geminiviruses, such as TYLCSV, can overcome resistance via silencing of transgenes (Noris *et al.,* 2004; Brunetti *et al.,* 1997; Lucioli *et al.,* 2003).

**Resistance due to RNA-silencing**

RNA silencing is a phenomenon in which expression of a gene is silenced by RNA molecules that are homologous to portions of the silenced gene (Almeida and Allshire, 2005). Transgenic plants showing RNA silencing usually contain multiple copies of transgenes (Goldbach *et al.,* 2003). The transcriptional rate of the transgene is usually high, but the steady-state mRNA level is usually low

(Longstaff *et al.,* 1993; Smith *et al.,* 1994; Swaney *et al.,* 1995). To trigger RNA silencing of a target gene in plants, it was previously believed that a transgene had to be > 300 nt in length, with > 90 % nt sequence identity with the targeted gene (Ritzenthaler, 2005; Chellappan *et al.,* 2004a). Nevertheless, in recent studies, short (21~26 nt) RNA interfering molecules (RNAi) also triggered complete silencing (Parizotto *et al.,* 2004; Wang and Metzlaff, 2005).

RNA silencing has been observed in plants transformed with genes from geminiviruses. RNA silencing is induced by dsRNA and mediated by the ribonuclease, Dicer (an RNase-III-like ribonuclease), RNA-dependent RNA polymerases (RDRs) and Argonautes (AGOs, RNase-H-like ribonucleases) (Bisaro, 2006). Four Dicer-like enzymes (DCL), six RDRs and ten AGOs have been found in *Arabidopsis thaliana* (Bartel, 2004; Meins *et al.,* 2005; Baulcombe, 2005; Akbergenov *et al.,* 2006; Baumberger and Baulcombe, 2005; Zilberman *et al.,* 2004; Voinnet, 2005). Unlike RNA viruses, replication of geminiviruses does not have a dsRNA phase. Sources of dsRNA to silence genes of geminiviruses can be folded inverted repeats or annealed complementary mRNA generated from viral genomes or transgenes (Gazzani *et al.,* 2004; Molner *et al.,* 2005). Host RDRs may also act on aberrant or over-abundant mRNA transcripts to produce dsRNA (Szittya *et al.,* 2002).

There are at least three RNA silencing pathways (Baulcombe, 2004; Vanitharani *et al.,* 2005). The first pathway is known as post-transcriptional gene silencing (PTGS) or cytoplasmic RNA silencing. The second pathway is short interfering RNA (siRNA)-directed transcriptional gene silencing (TGS). The third pathway is microRNA (miRNA)-directed gene silencing.

In PTGS, inducing dsRNAs are degraded into duplex 21 and 22nt siRNAs, most likely by DCL-2 and DCL-4 (Gasciolli *et al.,* 2005; Xie *et al.,* 2004). The duplex siRNA is then unwound and one strand incorporates with AGO to form an RNA-induced silencing complex (RISC) (Hammond *et al.,* 2000; Hannon, 2002). The siRNAs in the RISC then hybridise to their complementary sequences in mRNAs, so AGO in RISC can degrade mRNAs with sequence specificity (Liu *et al.,* 2004). Eventually, host RDRs amplify siRNAs that spread from cell-to-cell systematically *via* vascular tissues to trigger PTGS in the whole plant (Palaqui *et al.,* 1997; Voinnet and Baulcombe, 1997). For example, evidence of PTGS, such as 22nt siRNA molecules and low mRNA levels, were detected in Cotton leaf curl virus (CLCuV)-resistant plants that had been transformed with the 5' or 3' half of the Rep gene of CLCuV in sense or anti-sense orientation (Asad *et al.,* 2003). Wild-type Rep gene of TYLCV, TGMV and ToLCV in an anti-sense orientation, as well as the partial Rep gene and the intergenic regions of TYLCV in sense or anti-sense, were all shown to either suppress the replication of, or the symptoms caused by, the viruses that the transgenes were originally derived from, presumably also by PTGS (Bendahmane and Gronenborn, 1997; Yang *et al.,* 2004; Day *et al.,* 1991; Praveen *et al.,* 2005; 2006). In addition, double-stranded 21 nt siRNAs with homology to part of the ACMV Rep mRNA, have been introduced directly into tobacco protoplasts and have successfully suppressed replication of the co-delivered ACMV genome; the mRNA level of the Rep gene was low in protoplasts which also suggested a PTGS mechanism (Vanitharani *et al.,* 2003).

Cassava plants transformed with sense-orientated sequences of wild-type Rep or a full-length Rep with a defective ATPase domain were highly resistant to

ACMV as was the case for the closely related *East African cassava mosaic Cameroon virus* (EACMCV) and *Sri Lankan cassava mosaic virus* (SLCMV) (Chellappan *et al.,* 2004a). The nucleotide sequences of the ACMV Rep are only 66 % and 67 % identical to the Rep genes of EACMCV and SLCMV, respectively (Chellappan *et al.,* 2004a). The results suggested that the observed broad-spectrum resistance may not result from RNA silencing, which generally requires > 90% homology between the transgene and the targeted gene (Chellappan *et al.,* 2004a). Features that are characteristic of RNA silencing, however, such as reduced mRNA level of Rep gene and siRNAs with sequences homologous to the mRNA of Rep, were observed in the resistant lines of transgenic cassava (Chellappan *et al.,* 2004a). It was suggested that, although the nucleotide sequences of the above Rep of viruses were not highly homologous, their mRNA transcripts could be digested into siRNAs of which a few would show 100% complementarities to portions of the mRNA of heterologous Rep genes (Chellappan *et al.,* 2004a). Interestingly, in the resistant cassava lines, siRNAs of 24-26 nt were found with 21-22 nt siRNAs (Chellappan *et al.,* 2004a). As mentioned earlier, the 21-22 nt siRNAs provided evidence of PTGS (Gasciolli *et al.,* 2005; Xie *et al.,* 2004). The 24-26 nt siRNAs were previously believed to be responsible for systemic spreading of PTGS signals (Hamilton *et al.,* 2002). However, Mallory *et al* (2003) reported that accumulation of 24-26 nt siRNAs in plants were not correlated with occurrence of systemic RNA silencing, but were found associated with a second pathway of RNA silencing, called transcriptional gene silencing (TGS).

TGS is often induced by genes encoded as inverted repeats or tandem repeats that can fold into dsRNAs (Jones *et al.,* 1999; 2001; Hamilton *et al.,* 2002; Qi *et al.,* 2005; Xie *et al.,* 2004; Zilberman *et al.,* 2003; Fuentes *et al.,* 2006). The dsRNAs are then degraded by DCL-3 into 24-26 nt siRNAs, that form RISC with AGO and are amplified by RDR2 (Jones *et al.,* 1999; 2001; Hamilton *et al.,* 2002; Qi *et al.,* 2005; Xie *et al.,* 2004; Zilberman *et al.,* 2003; Fuentes *et al.,* 2006). The slightly larger siRNAs that result could lead to methylation of cytosine residues in DNA and thus silence transcription (Lippman and Martienssen, 2004). Also, indirect evidence also suggests that, in geminiviruses, siRNAs can induce hyper-methylation of the promoters to down-regulate transcription of viral genes (Seemanpillai *et al.,* 2003; Pooggin and Hohn, 2004).

When both 21-22 nt and 24-26 nt siRNAs are found, as in the case of Chellappan *et al* (2004a), both PTGS and TGS could have contributed to gene silencing. In some cases where only 24-26 nt siRNA are found, TGS is likely to be the sole mechanism involved. For example, only 24 nt siRNAs were observed in ACMV resistant lines of cassava that were transformed with the full-length Rep gene in anti-sense orientation (Zhang *et al.,* 2005). Another example comes from a study by Fuentes *et al* (2006). They transformed tomato plants with a "stem-loop" construct that had castor bean catalase intron as the loop and two 726 nt fragments representing the 3' half of TYLCV Rep gene, one at each side of the loop, as the stem; and only ~ 25 nt siRNAs were observed in TYLCV-resistant lines (Fuentes *et al.,* 2006).

The third pathway of RNA silencing is mediated by RISC formed with miRNAs of 21-22 nt and AGOs (Llave *et al.,* 2002; Baumberger and Baulcombe,

2005). The miRNAs are excised by DCL-1 from stem-loops of larger miRNA precursors encoded by non-protein-coding genes (Kurihara and Watanabe, 2004; Bartel, 2004; Qi *et al.,* 2005; Xie *et al.,* 2004). This pathway has not been exploited to generate PDR against geminiviruses.

Many geminiviruses encode RNA silencing suppressors such as the AL2 (sometimes also known as AC2 or C2) and AL4 (sometimes also known as AC4 or C4), and these can interfere with PDR-mediated RNA silencing. Proteins encoded by AL2 and AL4 suppress RNA silencing via different mechanisms. AL2 proteins interfere with the TGS pathway by inhibiting the adenosine kinase (ADK) of the methylation cycle (Bisaro, 2006). Interestingly, TYLCV encodes a TGS suppressor (C2) gene, but TGS of the Rep of TYLCV has been observed (Fuentes *et al.,* 2006). Results suggest that the C2 gene may not be activated every time that TGS occurs. The AL4 ORFs are often found internal to the Rep ORF of geminiviruses, but many AL4 ORFs encode proteins of unknown function. AL4 proteins that are RNA silencing suppressors interfere with the RISC functions by binding to single-stranded siRNA or miRNA (Bisaro, 2006). For example, TYLCSV has been shown to overcome transgene-mediated RNA silencing of the Rep gene possibly due to activities of its C4 gene that potentially encodes an RNA silencing suppressor (Noris *et al.,* 2004).

**Resistance against nanoviruses**

Rep-mediated resistance has not been investigated in nanoviruses. A mild strain of BBTV (strain TW4) however, that has a putative defective interfering (DI) DNA associated with its genome, has been found in Taiwan (Su *et al.,* 2003). This DI is 537 nt in length and encodes a Rep protein that contains a deletion of

556 nt within the ATPase domain. This Rep mutant may interfere with BBTV replication, leading to mild symptoms associated with the TW4 strain. The observation suggests that T-Rep or mutated Rep without ATPase functions has the potential to confer PDR against BBTV infection.

Furthermore, BBTV DNA-S1, which is a satellite DNA of BBTV, has also been shown to suppress replication of BBTV DNA-R, -S and -C in transiently transformed banana embryogenic cell suspensions (Horser *et al.*, 2001a). BBTV DNA-S1 encodes a Rep that is similar, but not identical, to the Rep encoded by BBTV DNA-R (Horser *et al.*, 2001b). The results suggest the similar Rep proteins may have interfered with each other to suppress replication of BBTV (Horser *et al.*, 2001a). Thus, the Rep of BBTV satellite DNAs may be another promising candidate for conferring resistance against BBTV.

Silencing suppressors have not been found in nanoviruses. Nevertheless, the small ORF (U5) in BBTV DNA-R with unknown function is located in a position equivalent to those AL4 ORFs that are located internally to the viral Rep gene. If the U5 ORF of BBTV encodes an RNA silencing suppressor, PDR mediated at the protein level could be more stable and a more desirable mechanism to generate resistance against BBTV.

### *2.2.2. Resistance mediated by CP-encoding genes*

Viral CPs form the capsid of viruses. Studies of CP-mediated resistance have mostly focused on RNA viruses. Plants transformed with CP genes from RNA viruses usually show specific viral resistance, which can be overcome by extremely high levels of inoculum, or inoculation of naked viral genomes

(Wilson, 1993; Beachy, 1994; Baulcombe, 1996; Goldbach *et al.,* 2003). Studies with RNA viruses suggest that the mechanism of CP-mediated resistance involves (1) interfering with virion disassembly in primary infected cells and (2) interfering with assembly and disassembly of later stages in the infection cycle such as during viral cell-to-cell movement (Register and Beachy, 1988; Reimann-Philipp and Beachy, 1993; Osbourn *et al.,* 1989; Clark *et al.,* 1995a; 1995b; Saito *et al.,* 1990; Wisniewski *et al.,* 1990; Bendahmane *et al.,* 2002).

There are only three reports of CP-mediated resistance against geminiviruses. Transgenic tomato plants that were transformed with the TYLCV CP gene showed delayed symptoms, followed by recovery when inoculated with TYLCV (Kunik *et al.,* 1994). The resistance operated strictly at the protein level, because expressing the untranslatable CP gene of TYLCV did not suppress disease symptoms (Kunik *et al.,* 1994).

Occasionally, the use of genes encoding viral CP generates viral resistance not by interfering with viral CP, but via PTGS of the viral CP gene (Sinisterra *et al.,* 1999; Andika *et al.,* 2005). For example, in transgenic tobacco transformed with mutated CP gene (with a 30 nt deletion at the 5' end), various levels of resistance against inoculation with ToMoV were observed and RNA from the CP gene, but not the protein, was detected in the resistant lines (Sinisterra *et al.,* 1999). PTGS was also responsible for resistance against *Beet necrotic yellow vein virus* (BNYVV) in transgenic *N. benthamiana* transformed with the CP gene (Andika *et al.,* 2005).

Other attempts to generate resistance to geminiviruses using CP genes have

failed. Recently, Shivaprasad *et al* (2006) transformed tobacco with the wild-type CP gene of *Mungbean yellow mosaic virus-Vigna* (MYMV-Vig), but accumulation of MYMV-Vig DNA was not suppressed in the transgenic tobacco.

### *2.2.3. Resistance mediated by genes encoding MPs or NSPs*

Viral MPs and NSPs often work in concert with each other in viral DNA transport (Sanderfoot *et al.,* 1996). Viral MPs are responsible for efficient cell-to-cell and long distance movement of viral DNA (Carrington *et al.,* 1996; Ding, 1998). Viral NSPs are responsible for intracellular movement of viral DNA from the nucleus to the cytoplasm (Sanderfoot *et al.,* 1996). MPs and NSPs have related functions and often behave in similar ways by conferring PDR against viruses, so that the two genes will be discussed jointly here.

Transgenic plants transformed with wild-type MP/NSPs are sometimes resistant to closely related viruses from the same genus but not to viruses belonging to other genera (Yoshikawa *et al.,* 2000). This type of resistance was presumably caused by inappropriate aggregation of related MPs/NSPs, which could lead to blockage of intercellular and intracellular trafficking of MP/NSPs (Isogai *et al.*, 2003).

Transgenic plants transformed with wild-type MP/NSPs are sometimes resistant against heterologous viruses but not to closely-related viruses (Heinlein *et al.,* 1995; 1998; McLean *et al.,* 1995; Reichel and Beachy, 1998; Reichel *et al.,* 1999). This type of resistance may be caused by competition between heterologous MP/NSPs over limited cellular factors, such as host membranes, cytoskeletal elements and for host proteins (Heinlein *et al.,* 1995; 1998; McLean

*et al.,* 1995; Reichel and Beachy, 1998; Reichel *et al.,* 1999). MP/NSPs do not function in a virus-specific manner and are inter-changeable among different viruses, which may explain broad-spectrum resistance (von Arnim and Stanley, 1992; Nejidat *et al.,* 1991; Solovyev *et al.,* 1996).

Defective MP/NSPs can generate resistance against both closely-related and unrelated viruses. Resistance against closely-related viruses may also be due to inappropriate aggregation with functional MP/NSPs and competition between the functional and defective MP/NSPs over cellular factors (Heinlein *et al.,* 1995; 1998; McLean *et al.,* 1995; Reichel and Beachy, 1998; Reichel *et al.,* 1999). Resistance against heterologous viruses may be due solely to competition for cellular factors (Heinlein *et al.,* 1995; 1998; McLean *et al.,* 1995; Reichel and Beachy, 1998; Reichel *et al.,* 1999).

Most studies of MP- or NSP-mediated resistance have focused on RNA viruses, although there have been several studies with DNA viruses. For example, transgenic tobacco plants transformed with a mutated full-length MP of ToMoV showed resistance against ToMoV and *Cabbage leaf curl virus* (CabLCV) infection (Duan *et al* 1997a). Transgenic tomato plants transformed with wild-type or mutated MP or NSP from *Bean dwarf mosaic virus* (BDMV) also showed delayed symptoms against ToMoV infection (Duan *et al.,* 1997a; 1997b; Hou *et al.,* 2000). Furthermore, the movement of ACMV was inhibited in transgenic *N. benthamiana* expressing the MP of TGMV (von Arnim and Stanley, 1992). The resistance mechanism against DNA viruses is presumably the same as for RNA viruses.

Despite the ability of MP/NSP transgenes to often confer broad-spectrum resistance, levels of resistance have never been sufficient for commercial use (Lapidot and Friedmann, 2002). For some geminiviruses, attempts to generate resistance using the viral MP/NSPs have failed completely. For example, tobacco plants transformed with wild-type MP gene from MYMV-Vig did not suppress the DNA accumulation of MYMV-Vig (Shivaprasad *et al.,* 2006). Further, expression of the MP from geminiviruses often induces symptom-like phenotypes in transgenic plants, making the approach undesirable (Hou *et al.,* 2000).

## 2.3. Conclusion

To date, there have been no reports of PDR against nanoviruses. However, based on studies of the putative DI DNA of BBTV (Su *et al.,* 2003) and BBTV DNA-S1 (Horser *et al.,* 2001a), Rep genes from BBTV and BBTV satellite DNAs have the potential to confer resistance against BBTV infection. To identify the most suitable BBTV gene constructs that could confer resistance against BBTV infection, the effect of BBTV satellite DNAs and their encoded Rep proteins on the replication of BBTV genome has been investigated in this thesis.

# CHAPTER 3

## EFFECT OF SATELLITE DNAS ON THE REPLICATION OF *BANANA BUNCHY TOP VIRUS*

# Abstract

# Abstract

The integral genome of *Banana bunchy top virus* (BBTV) consists of at least six circular, single-stranded (css) DNA components, namely BBTV DNA-R, -U3, -S, -M, -C and -N, that are found consistently in all geographical isolates of the virus. Additional css satellite DNAs, namely BBTV DNA-S1, -S2, -W2, -S3, -Y and -W1, are found occasionally in some Asian isolates of the virus. Both BBTV DNA-R and the BBTV satellite DNAs encode a replication initiation protein (Rep) and have been shown to self-replicate previously. However, only DNA-R that encodes the master Rep (M-Rep) can initiate replication of the BBTV integral genome components. This study investigated the effect of two BBTV satellite DNAs on replication of the BBTV integral genome. A new satellite DNA, designated BBTV DNA-S4, was cloned from a Vietnamese BBTV isolate. When 1.1mers of DNA-R, -C and -S4 were co-bombarded into banana embryogenic cells, replication of DNA-R was enhanced by DNA-S4 on Days 8 and 16, post-bombardment. Further, when the replicative capabilities of BBTV DNA-S4 and the previously characterised DNA-S1 were compared, the amount of DNA-S4 accumulated to higher levels than for DNA-S1. Consistent with previous studies, co-bombardment of banana embryogenic cell suspensions with 1.1 mers of BBTV DNA-S1 was found to suppress replication of DNA-R. In contrast, over-expression of the Rep encoded by DNA-S1 using the strong constitutive maize polyubiquitin promoter resulted in enhanced replication of DNA-R in bombarded banana cells.

# Introduction

*Banana bunchy top virus* (BBTV) is one of the most destructive viruses that infects banana (Dale, 1987). BBTV is the type member of the genus *Babuvirus* within the family *Nanoviridae* (Vetten *et al.,* 2005). The integral genome of the virus contains at least six circular, single-stranded (css) DNA components, named BBTV DNA-R, -U3, -S, -M, -C and -N (Burns *et al.,* 1994; Vetten *et al.,* 2005). Additional cssDNAs, named BBTV DNA-S1, -S2, -W2, -S3, -Y and -W1, have also been found occasionally with some Asian isolates of the virus (Horser *et al.,* 2001b; Wu *et al.,* 1994; Bell *et al.,* 2002; Yeh *et al.,* 1994). These additional DNAs have been termed satellite DNAs due to their restricted geographic distribution and different genome organisation compared with the integral BBTV DNA components (Horser *et al.,* 2001b).

BBTV DNA components are all ~ 1 kb in size and replicate by a rolling circle mechanism (Burns *et al.,* 1995; Hafner *et al.,* 1997a; 1997b). The genome organisation of the integral DNA components is similar - they contain (i) a single open-reading frame (ORF) (except DNA-R), (ii) an intergenic region containing conserved iterated sequences (iterons) and a stem-loop common region (CR-SL) containing the replication origin, (iii) a major common region (CR-M) located 5' of the CR-SL, and (iv) a polyadenylation signal 3' to the ORF (Burns *et al* 1995; Beetham *et al.,* 1999). DNA-R has two ORFs; the large ORF encodes a replication initiation protein (Rep), but the function of the gene product (U5) encoded by the small, internal ORF is unknown (Hafner *et al.,* 1997b; Beetham *et al.,* 1997). The function of DNA-U3 is also unknown, while DNA-S, -M, -C and -N encode the coat protein, movement protein, cell-cycle link (Clink) protein and

the nuclear shuttle protein, respectively (Wanitchakorn *et al.,* 1997; 2000).

BBTV satellite DNAs each contain one large ORF encoding a Rep but, unlike BBTV-R, do not contain a small, internal ORF. The genome organisation of BBTV satellite DNAs is also different to that of the integral BBTV DNA components; (i) the sequences of the iterons in the CR-SL are different, (ii) the potential TATA boxes are located 5' of the stem-loop structure and (iii) the CR-M is found only in DNA-S1, but not in DNA-S2, W2, -S3, -Y nor -W1 (Horser *et al.,* 2001b; Bell *et al.,* 2002). Phylogenetic studies showed that the BBTV satellite DNAs are more closely related to each other than to BBTV DNA-R or the satellite DNAs of the nanoviruses, *Milk vetch dwarf virus* (MDV) or *Subterranean clover stunt virus* (SCSV) (Bell *et al.,* 2002).

The replicative capabilities of different BBTV genomic components have been investigated using greater-than-unit-length artificial constructs that contain two intergenic regions of the viral genome (i.e. BBTV 1.1mers). Horser *et al.* (2001a) used plasmid clones of 1.1mers to study the rolling circle replication of BBTV DNA-R and -S1 for a period of up to eight days. They found that DNA-S1 was capable of self-replication in banana embryogenic cell suspensions, but was unable to initiate replication of BBTV DNA-S and -C (Horser *et al.,* 2001a). Replication of DNA-S1 was also shown to be enhanced in the presence of DNA-R, -S and -C (Horser *et al.*, 2001a). Interestingly, replication of DNA-R, -S and -C was shown to be weakly suppressed by DNA-S1 on Day 4 and 8 post-bombardment (Horser *et al.,* 2001a). Although this latter result was preliminary and needed further investigation, the effect of DNA-S1 on replication of BBTV highlighted the possible use of this satellite DNA as a transgene to

generate pathogen-derived resistance to BBTV.

In this study, we extended the investigation into the interaction between DNA-R and -S1 by (i) examining the effect of over-expressing the ORF of DNA-S1, (ii) using a larger data set and (iii) extending the study for a period of up to 16 days. Further, banana plantlets were stably transformed with the plasmid clone of DNA-S1 1.1mer as a possible means of generating transgenic control of BBTV. We were also interested to determine the effect that other BBTV satellite DNAs had on replication of BBTV. As such, we isolated and characterised a Rep-encoding satellite DNA component from a Vietnamese isolate of BBTV and examined the replicative capability of this component and its effect on the replication of BBTV DNA-R.

# Materials and methods

## *Amplification of BBTV satellite DNAs*

Satellite DNAs were PCR-amplified from BBTV sample B1 (Bell *et al.,* 2002) using various primer pairs including TTTP24/TTTP25, S4-B2B-R258/S4-B2B-F263, TTTP6/TTTP9 and TTTP7/TTTP8 (Table 3-1). The B1 template comprised total DNA extracted from the leaf tissue of a BBTV-infected banana plant collected in Vietnam. The PCR mixture contained 1x Expand PCR Buffer 1 (Roche), with 10 pmol of primer pair, 200 μM dNTPs, 1 U Expand DNA polymerase (Roche), and 0.1 μg B1 DNA extract. The mixture was heated at 92 °C for 2 min, followed by 35 cycles of 92 °C for 30 sec, 55 °C for 30 sec and 68 °C for 90 sec, followed by 1 cycle of 72 °C for 10 min. The PCR amplicons were electrophoresed through 1 % agarose and DNA fragments of the expected sizes were excised and purified using a QIAquick PCR Purification Kit (QIAGEN). The DNA was ligated into pGEM-T (Promega), transformed into *Escherichia coli* DH5α and plasmids were extracted from selected clones and sequenced.

## *Generation of constructs*

### pBT1.1-R, pBT1.1-C and pBT1.1-S1

Constructs comprised 1.1mers of BBTV DNA-R (GenBank Accession No. NC_003479), -C (GenBank Accession No, NC_003477) and -S1 (GenBank Accession No. AF216221), respectively, ligated into pGEM-T (Promega) (Fig. 3-1), and were generously provided by Dr. Cathryn Horser (Horser *et al.,* 2001a). Plasmids pBT1.1-R and pBT1.1-C were previously designated pBT1.1-1 and pBT1.1-5, respectively (Horser *et al.,* 2001a).

**Table 3-1. List of Primers.**

| Name | Sequence* (5' to 3') | Location (nt) |
|---|---|---|
| ORF1F | ATGGCGCGATATGTGGTATGCT | 129-149 of DNA-R |
| ORF1R | TCAGCAAGAAACCAACTTTAT | 989-968 of DNA-R |
| Sma_Bam_S1_F | **CCCGGGGGATCC**ATGTCATCCTTTAAATGGTG | 63-82 of DNA-S1 |
| Sma_Kpn_S1_R | **CCCGGGGGTACC**TCAGCAATAAATGATTTTTATTCC | 917-894 of DNA-S1 |
| TTTP24 | ATGTCATCCTTTAAATGGTGC | 63-83 of DNA-S1 |
| TTTP25 | TCAGCAATAAATGATTTTTATTCC | 917-894 of DNA-S1 |
| TTTP6 | TTTATAGGTGCGCGGAGGTAG | 49-29 of DNA-S3 |
| TTTP7 | ACACGCTATGACAAACGCAC | 902-921 of DNA-S3 |
| TTTP8 | CGTTTTCGTATTAGCGGAATA | 305-286 of DNA-S3 |
| TTTP9 | ACGCCTTGTTGTCGTAGTCT | 208-238 of DNA-S3 |
| TTTP20 | ATGTCTGCCTCTCGTTGGACA | 50-70 of DNA-S3 |
| TTTP21 | TCAGCAAGCAACTATTTTTATTC | 901-879 of DNA-S3 |
| S4-B2B-R258 | GTACGCCTTGTTGTCGTAGTC | 258-238 of DNA-S4 |
| S4-B2B-F263 | CCAAAGAAGCCCTAATTCTTG | 263-283 of DNA-S4 |

**Sequences in bold are the restriction sites.*

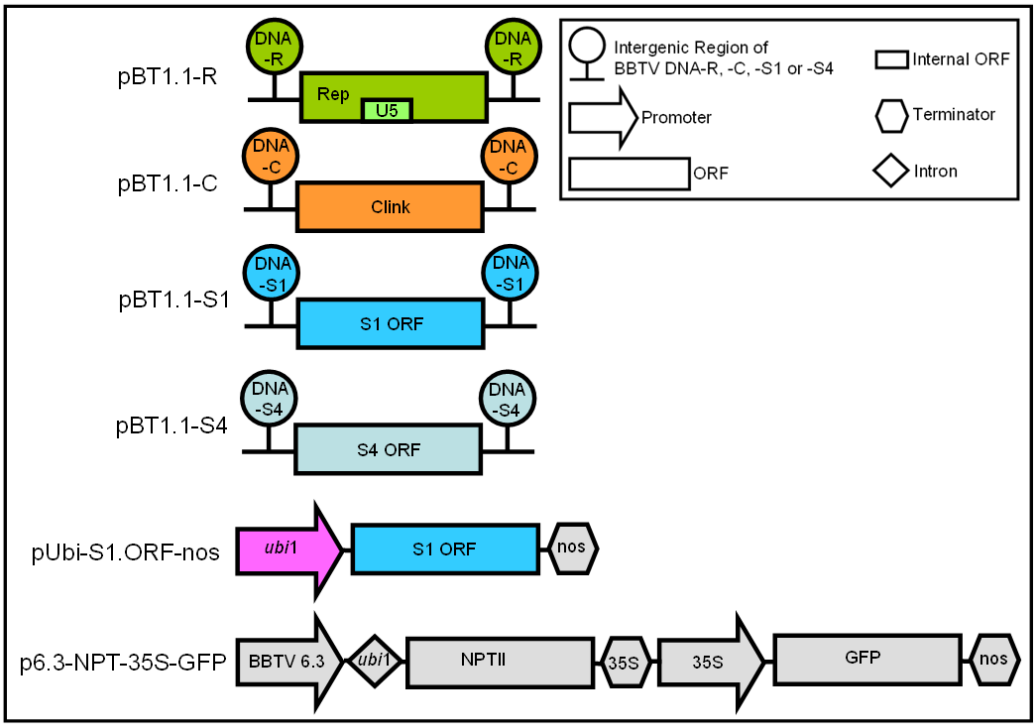



**Fig. 3-1. Maps of constructs**

The *ubi*1 promoter and intron are the promoter and intron of maize polyubiquitin 1 gene. The *nos* terminator is the terminator sequence of a nopaline synthase. The BBTV 6.3 promoter was derived from BBTV DNA-N (Dugdale *et al.*, 1998). The NPTII gene encodes the neomycin phosphotransferase II. The CaMV 35S promoter and terminator are the promoter and terminator sequences from *Cauliflower mosaic virus* (CaMV). The GFP gene encodes the green fluorescent protein from *Aequorea victoria*.

**pBT1.1-S4**

A plasmid clone of DNA-S4 1.1mer (Fig. 3-1) was constructed using a PCR-based strategy, in which two overlapping fragments were amplified, digested and ligated into pGEM-T. The PCR mixture contained 1 x Expand PCR Buffer 1 (Roche), with 10 pmol of primer (TTTP6/TTTP9 and TTTP7/TTTP8), 200 μM dNTPs, 1 U Expand DNA polymerase (Roche) and 0.1 μg B1 DNA extract. The PCRs were performed as previously described for amplification of BBTV satellite DNAs. The overlapping amplicons generated with primer pairs TTTP6/TTTP9 and TTTP7/TTTP8 were digested with XhoI, and electrophoresed through 1 % agarose. DNA fragments of the expected sizes were excised, purified and ligated together into pGEM-T (Promega). The ligation was transformed into *E. coli* DH5α and plasmids were extracted from selected clones and sequenced.

**pUbi-S1.ORF-nos**

The ORF of DNA-S1 was amplified from pBT1.1-S1 using primers Sma_Bam_S1_F and Sma_Kpn_S1_R (Table 3-1) in a PCR. The reaction mixture consisted of 10 pmol of each primer, 200 μM dNTPs, 1 U Expand DNA polymerase (Roche), and 0.1 μg of pBT1.1-R (dissolved in sterilised $H_2O$) in 1 x Expand PCR Buffer 1 (Roche). The reaction mixture was heated at 92°C for 2 min, followed by 35 cycles of 92 °C for 30 sec, 50 °C for 30 sec and 68 °C for 90 sec, followed by 1 cycle of 72 °C for 10 min. The PCR products were ligated into pGEM-T at 14 °C for 16 hours using 2 U of T4 DNA ligase (Roche) and the ligations were transformed into *E. coli* DH5α. Plasmids were extracted from selected clones, digested with KpnI and BamHI, electrophoresed through 1 % agarose gels in TAE buffer and stained with ethidium bromide. Inserts were excised and purified from the gel using a High Pure Gel Extraction Kit (Roche).

The DNA-S1 ORF fragment was inserted into the BamHI/KpnI sites located between the *ubi*1 (maize polyubiquitin 1) promoter and *nos* (nopaline synthase) terminator, in the plasmid pGEM-*ubi*-*nos*.

**p6.3-NPT-35S-GFP**

The construct (Fig. 3-1) was already available from previous work and was generously provided by Mr. Matthew Webb (QUT). It comprised the BBTV 6.3 promoter-*ubi*1 intron-NPTII-CaMV 35S terminator together with the CaMV 35S promoter-GFP-*nos* terminator. This plasmid was used as a "stuffer" construct to ensure equal molar amounts of DNA were used in experiments. Further, the plasmid acted as a selectable marker gene (NPTII) for generating stable transformants and a reporter gene (GFP) in microprojectile bombardment.

## *Sequencing*

All constructs were purified using a BRESA-pure MAXi-prep Plasmid Purification kit (Geneworks). Constructs were sequenced using an automatic sequencer and Big Dye Termination Cycle Sequencing Ready Reaction V3.1 (PE Applied Biosystems). Primers used for sequencing included specific primers listed in Table 3-1 and M13 universal sequencing primers (US Biochemical).

## *Sequence analysis*

Amino acid (aa) and nucleotide (nt) sequences were compared using DNASTAR MegAlign software. Sequences were compiled into a consensus sequence using DNASTAR SeqMan software. The nt sequences of several Rep-encoding DNA components of nanoviruses and geminiviruses were aligned and bootstrapped by Clustal X software version 1.8 (Thompson *et al.*, 1997). The

output of this phylogenetic analysis was constructed into an unrooted neighbor-joining tree using TreeView software version 1.6.1 (Page, 1996).

### *Microprojectile bombardment*

Banana "Lady Finger" (*Musa* spp. AAB group) somatic embryogenic cell suspension cultures were prepared and maintained by Ms. Jennifer Kleidon (QUT) as described in Khanna *et al.* (2004). Somatic embryos were harvested and approximately 0.1 g of condensed cell suspensions were plated onto filter papers and placed on solid Bluggoe Low culture media (Dheda *et al.,* 1991). Each plate was bombarded with various combinations of DNA constructs (1 μg each) using a particle inflow gun and gold microcarriers (BioRad) essentially as described by Dugdale *et al.* (1998).

### *Transient transformation*

On Day 4, 8, 12, 16 or 20 post-bombardment, transformation efficiency was monitored by observing GFP expression in cells using a Leica MZ12 stereo microscope with GFP-Plus fluorescence module and green barrier filter (BGG22, Chroma Technology). Cell samples were also collected on these days. Cells from different plates were stored in Eppendorf tubes at -80°C prior to testing.

### *Stable transformation*

The plasmids pBT1.1-S1 (1 μg) and p6.3-NPT-35S-GFP (1 μg) were co-bombarded into banana embryogenic cell suspensions. Cells were regenerated essentially as described in Becker *et al* (2000) on selective culture media containing kanamycin. Leaf tissues were collected from the plantlets and stored at -80 °C prior to analysis.

## *Plant DNA extraction*

Total nucleic acids from transformed and untransformed banana cells were extracted and resuspended in TE buffer (pH 8) essentially as described by Stewart and Via (1993). RNA was removed by RNase A digestion and DNA was quantified by spectrophotometry (Sambrook and Russell, 2000).

## *Generation of digoxigenin (DIG)-labeled probes*

The ORFs of BBTV DNA-R (DIG-ORF-R), DNA-S1 (DIG-ORF-S1) and DNA-S4 (DIG-ORF-S4) were used as probes. DIG-ORF-R was PCR amplified from pBT1.1-R using primers ORF1F and ORF1R (Table 3-1), DIG-ORF-S1 was amplified from pBT1.1-S1 using primers TTTP24/TTTP25 while DIG-ORF-S4 was amplified from pBT1.1-S4 using primers TTTP20/TTTP21. The PCRs were done as previously described for amplification of the ORF of DNA-S1. Amplicons were electrophoresed through 1% agarose gels in TAE buffer, pH 7.8 and stained with ethidium bromide. Fragments of the expected size (~ 850 bp) were excised and purified from the gel using a High Pure Gel Extraction Kit (Roche). These fragments were used subsequently as the template for a second round of PCR, with the dNTPs replaced with 5 μl DIG labelling mix (Roche).

The probes were purified using a QIAquick PCR purification kit and their concentration was quantified by spectrophotometry (Sambrook and Russell, 2000), while signal strength and incorporation of DIG-labelled nucleotides were compared *via* dot blots (Sambrook and Russell, 2000). Plasmids pBT1.1-R, pBT1.1-S1 and pBT1.1-S4 were denatured in TE buffer (pH 8) by boiling for 5 min and spotted onto positively charged nylon membranes (Roche). The membranes were baked at 80 °C for 2 hours, pre-hybridised in DIG-Easy Hyb

(Roche) for 1-2 hours and hybridised with either (i) a mixture of denatured DIG-ORF-S1 and DIG-ORF-S4 (250 nmol each) or (ii) DIG-ORF-S4 (250 nmol) in 10 ml of DIG-Easy Hyb at 42 °C for 12-16 hours, followed by high stringency washes (0.1 x SSC, 0.1 % SDS) at 65 °C prior to development as per the manufacturer's instructions (Roche).

### *Analysis of transient transformants*

The accumulation of BBTV DNA-R, -S1 and -S4 in bombarded cells was studied by Southern analysis and was taken to indicate the abundance of replication of these BBTV components. Total nucleic acids were extracted from bombarded banana embryogenic cell suspensions and 20 μg was electrophoresed through 1.5% agarose gels in 1 x TAE buffer (pH 7.8), and stained with ethidium bromide. As replicates for each experiment, three cell cultures were independently bombarded with each combination of constructs. Total nucleic acid (20 μg) from untransformed banana embryogenic cells was also included as a negative control. Sizes of DNA on the agarose gels were determined by comparison with DIG-labelled molecular marker III (Roche). Nucleic acids were transferred from the agarose gel to positively charged nylon membranes (Roche) after 16 hours of capillary blotting (Southern, 1975). Nylon membranes were baked at 80 °C for 2 h, pre-hybridised in DIG-Easy Hyb (Roche) at 42 °C for 1-2 h, hybridised with 250 nmol of DIG-ORF-R, DIG-ORF-S1 or DIG-ORF-S4 in 10 ml of DIG-Easy Hyb at 42 °C for 16 h, and exposed to X-Ray films (AGFA). The X-Ray films were developed by automatic developer (AGFA). Densitometry of the DIG signals was analysed by TotalLab version 1.11 (Phoretix). The quantitative densitometry data was analysed on Microsoft Office Excel 2003 version SP2 (Microsoft) using type 3 of the 2-tailed *t*-test, which did not assume homogeneity of variances.

## *Analysis of stable transformants*

### Presence of transgene

Total nucleic acid was extracted from the leaves of transformed plants and 0.1 μg was used as the template to amplify the ORF of DNA-S1, using primers TTTP24 and TTTP25, as previously described.

### Southern analysis

Nucleic acids (10 μg) extracted from each of the transformed plants was electrophoresed through a 1.5 % agarose gel in 1x TAE buffer (pH 7.8) and stained with ethidium bromide. Negative and positive controls (i.e. 10 μg DNA from untransformed banana leaf tissue and 1 μg pBT1.1-S1) were also loaded onto gels. A DIG-labeled molecular marker III (Roche) was also included for size comparisons. Nucleic acids were transferred from the gel to positively charged nylon membranes (Roche) by capillary blotting (Southern, 1975). Membranes were baked, pre-hybridised, and hybridised with 250 nmol DIG-ORF-S1 probe as previously described.

# Results

# Results

### *BBTV DNA-S1 suppressed the replication of DNA-R*

Banana embryogenic cell suspensions were bombarded with 1.1mers of BBTV DNA-R (pBT1.1-R) and DNA-C (pBT1.1-C), in combination with 1.1mers of DNA-S1 (pBT1.1-S1) or the stuffer construct p6.3-NPT-35S-GFP. Total DNA was extracted from each sample on Day 4, 8 and 16 post-bombardment, Southern blotted and hybridised initially with DNA-R specific probes. Blots were then stripped and hybridised with DNA-S1-specific probes. DNA samples loaded onto the same gel were extracted from the same batch of cultured and bombarded cell suspensions. Replication was assessed qualitatively by presence of the different conformational forms of BBTV genomic DNA including open circular, linear and supercoiled, in addition to multimeric intermediates resulting from rolling-circle replication. Identities of the DNA conformations were based on reference to molecular weight markers and from previous studies (Horser *et al.,* 2001a). Replication was assessed quantitatively using densitometry readings based on the supercoiled, replicative episomal forms of DNA-R or DNA-S1.

When 1.1mers of BBTV DNA-R and -C were bombarded into embryogenic cells, DNA-R specific bands were detected on Day 4, 8 and 16 post-bombardment (Fig. 3-2a) indicating that replication of this component had occurred as expected. Although various conformations of viral DNA were observed, supercoiled DNA appeared to the most abundant conformation in each sample. When 1.1mers of BBTV DNA-R and -C were co-bombarded with 1.1mers of DNA-S1, DNA-R

specific bands were also detected on Day 4, 8 and 16 post-bombardment (Fig. 3-2a). However, when the signal intensities of the supercoiled forms of DNA in

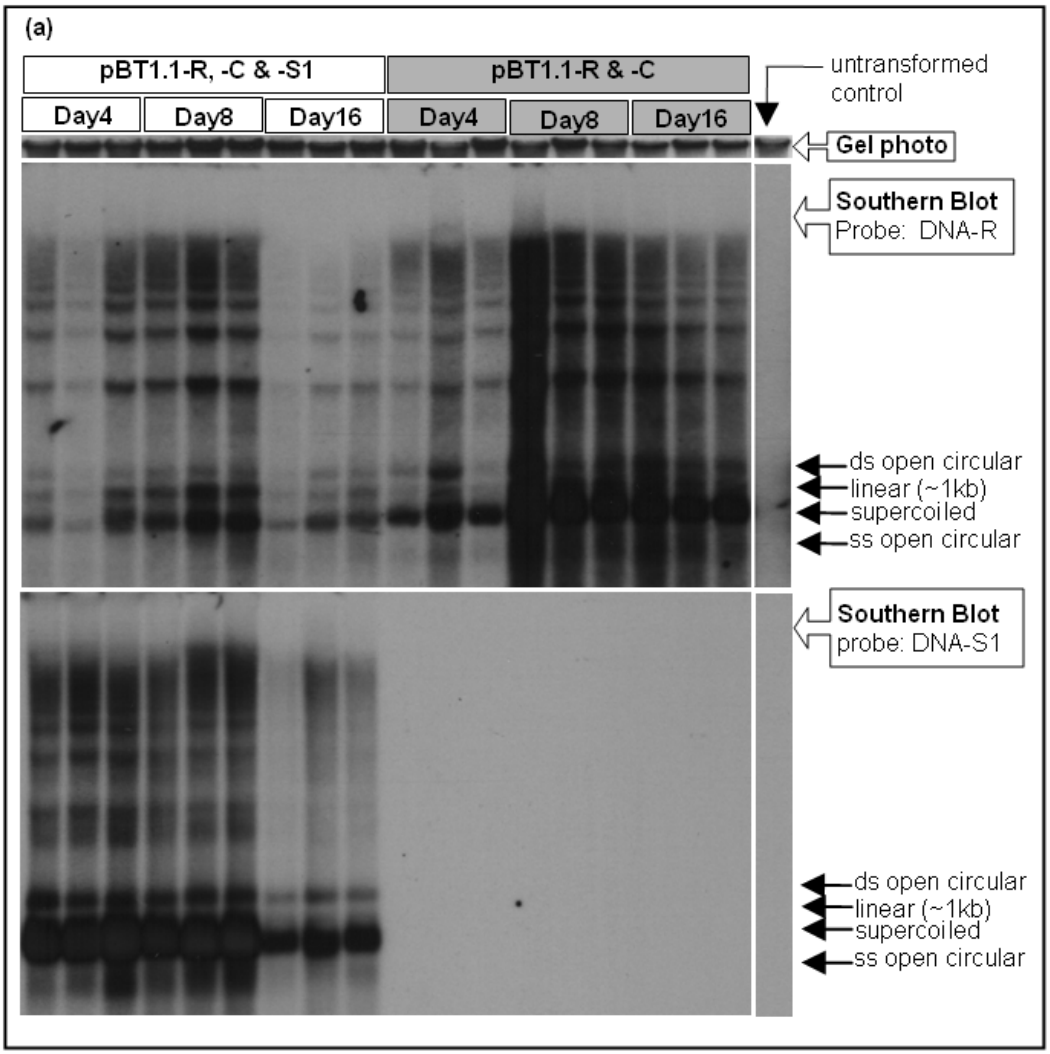



**Fig. 3-2. Replication of BBTV DNA-R and -S1 in banana embryogenic cell suspensions**

**(a)** pBT1.1-R, pBT1.1-C and pBT1.1-S1 were co-bombarded into banana embryogenic cell suspensions. The replication of DNA-R and -S1 in the cells were examined on Day 4, 8 and 16 post-bombardment by Southern blots using the DIG-ORF-R or DIG-ORF-S1 probes specific to the ORFs of DNA-R or -S1. Three replicates are shown for each time point. The gel photo shows equal amount of undigested DNA, extracted from the bombarded cells, was loaded to each lane. The blots were exposed to X-ray films for 2 hours.

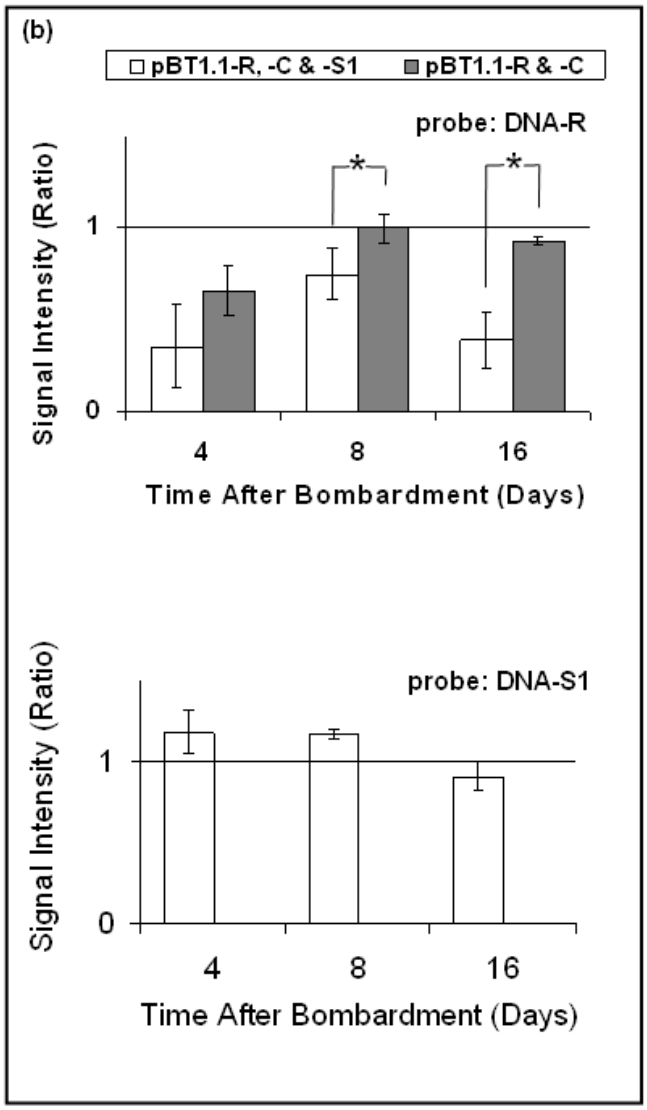



**Fig. 3-2. Replication of BBTV DNA-R and -S1 in banana embryogenic cell suspensions**

**(b)** Southern signal intensities for ss supercoiled DNA-R and -S1 were quantified. The mean signal intensities for samples collected at each time point are represented as rectangles. The "*" sign indicates significantly difference ($P \leq 0.05$) between the two rectangles below each "*" sign. The error bars indicates the 95 % confidence intervals. The line across the graph demarcrates the ratio of 1, which is the mean value of the three samples co-bombarded by pBT1.1-R and pBT1.1-C, collected on Day 8 and hybridised with the DIG-ORF-R probe in Southern analyses. The ratio of 0 is the signal intensity of the untransfromed control.

each experiment were quantitated by densitometry and analysed statistically (Fig. 3-2b), DNA-S1 was shown to weakly suppress the replication of DNA-R on Day 8 (P = 0.05) and significantly suppress the replication of DNA-R on Day 16 post-bombardment (P = 0.02).

## *Over-expression of the DNA-S1 ORF enhanced the replication of BBTV-R*

To determine the effect of over-expression of the DNA-S1 ORF on the replication of DNA-R, banana cell suspensions were co-bombarded with 1.1mers of DNA-R and DNA-C, with stuffer construct or with pUbi-S1.ORF-nos, a construct in which the expression of the DNA-S1 ORF was controlled by the strong, constitutive *ubi*1 promoter. Total DNA was extracted from each sample, electrophoresed through agarose, Southern blotted and hybridised with a DIG-ORF-R specific probe (Fig. 3-3a). The signal intensities of supercoiled DNA-R on the blots were quantitated by densitometry and analysed statistically (Fig. 3-3b). Although there was no significant effect of pUbi-S1.ORF-nos on DNA-R accumulation on Day 4 (P = 0.42), pUbi-S1.ORF-nos was shown to significantly enhance replication of DNA-R on Day 8 (P = 0.03) and Day 16 (P = 0.01).

## *Generation and characterisation of BBTV DNA-S1 transgenic banana plantlets*

To generate banana plants stably transformed with pBT1.1-S1, banana cell suspensions were co-bombarded with pBT1.1-S1 and the selective marker construct p6.3-NPT-35S-GFP. Transformants were regenerated into plantlets on selective media containing kanamycin. The presence of the DNA-S1 transgene

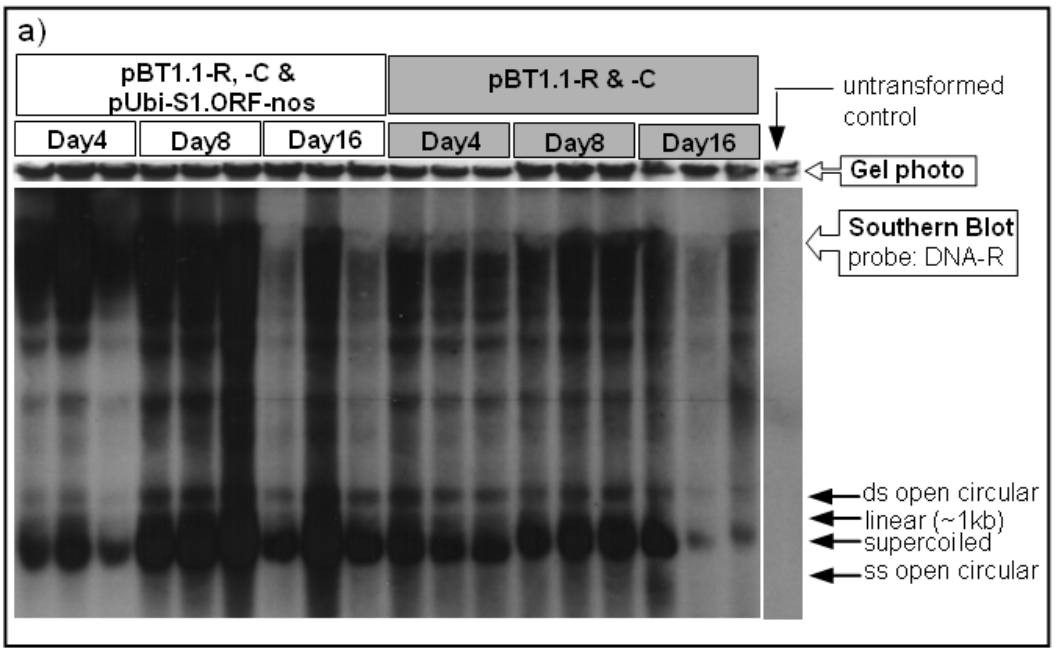



**Fig. 3-3. Replication of BBTV DNA-R in banana embryogenic cell suspensions**

**(a)** pBT1.1-R, pBT1.1-C and pUbi-S1.ORF-nos were co-bombarded into banana embryogenic cell suspensions. The replication of DNA-R in the cells were examined on Day 4, 8 and 16 post-bombardment by Southern blots using the DIG-ORF-R probes specific to the ORF of DNA-R. Three replicates are shown for each time point. The gel photo shows equal amount of undigested DNA, extracted from the bombarded cells, was loaded to each lane. The blots were exposed to X-ray films for 2 hours.

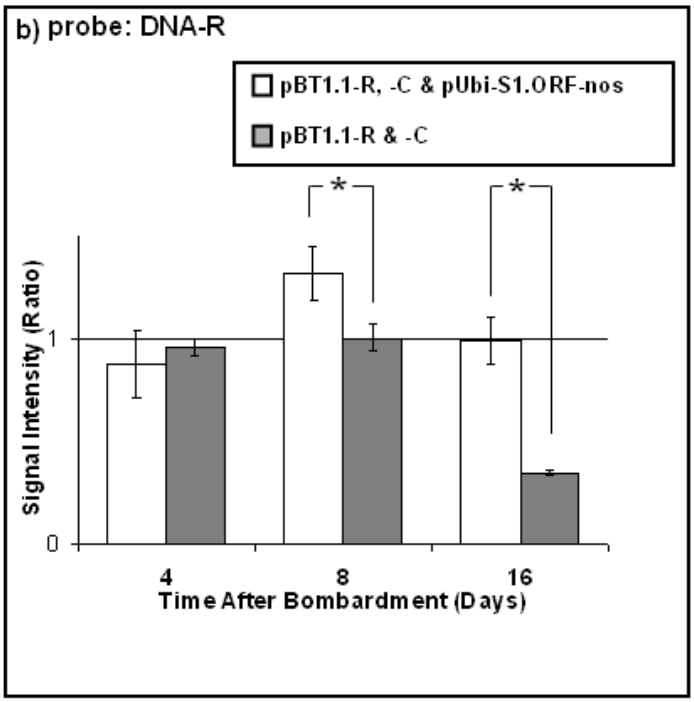



**Fig. 3-3. Replication of BBTV DNA-R in banana embryogenic cell suspensions**

**(b)** Southern signal intensities for ss supercoiled DNA-R were quantified. The mean signal intensities for samples collected at each time point are represented as rectangles. The "*" sign indicates significantly difference ($P \leq 0.05$) between the two rectangles below each "*" sign. The error bars indicates the 95 % confidence intervals. The line across the graph demarcates the ratio of 1, which is the mean value of the three samples co-bombarded by pBT1.1-R and pBT1.1-C, collected on Day 8 and hybridised with the DIG-ORF-R probe in Southern analyses. The ratio of 0 is the signal intensity of the untransformed control.

was verified in seven lines by PCR; these transgenic plantlets all appeared phenotypically normal.

Undigested total DNA was extracted from leaves of the seven transgenic plantlets, electrophoresed through agarose and Southern blotted. The blots were hybridised with probes specific for the ORF of DNA-S1. Although no replicative forms of DNA-S1 were observed, larger sized (> 10 kb) DNA-S1 specific signals were present (data not shown), suggesting the DNA-S1 sequence was incorporated into the plant genome.

## *Characterisation of BBTV DNA-S4*

Initially, primer pairs TTTP6/TTTP9 and TTTP7/TTTP8 were used in an attempt to amplify the previously characterised DNA-S3 (GenBank accession no. AF416471) from a Vietnamese BBTV DNA extract (BBTV sample no. B1, collected by Bell *et al.,* 2002). Amplicons of the expected sizes were generated and these were cloned and sequenced. Analysis of a full-length sequence revealed that it was similar, but not identical, to DNA-S3; this potentially new satellite DNA was designated BBTV DNA-S4. To confirm the presence of DNA-S4, specific primer pairs TTTP24/TTTP25 and S4-B2B-R258/S4-B2B-F263 were used in a PCR with the same DNA extract. The resulting amplicons were cloned and six clones of each amplicon were sequenced. The 12 sequences were compiled into an 1103 nt consensus sequence. The nt sequence of DNA-S4 and the aa sequence of the putative protein encoded by the ORF are illustrated in Fig. 3-4a.

DNA-S4 contained a single ORF comprising 855 nt which encoded a

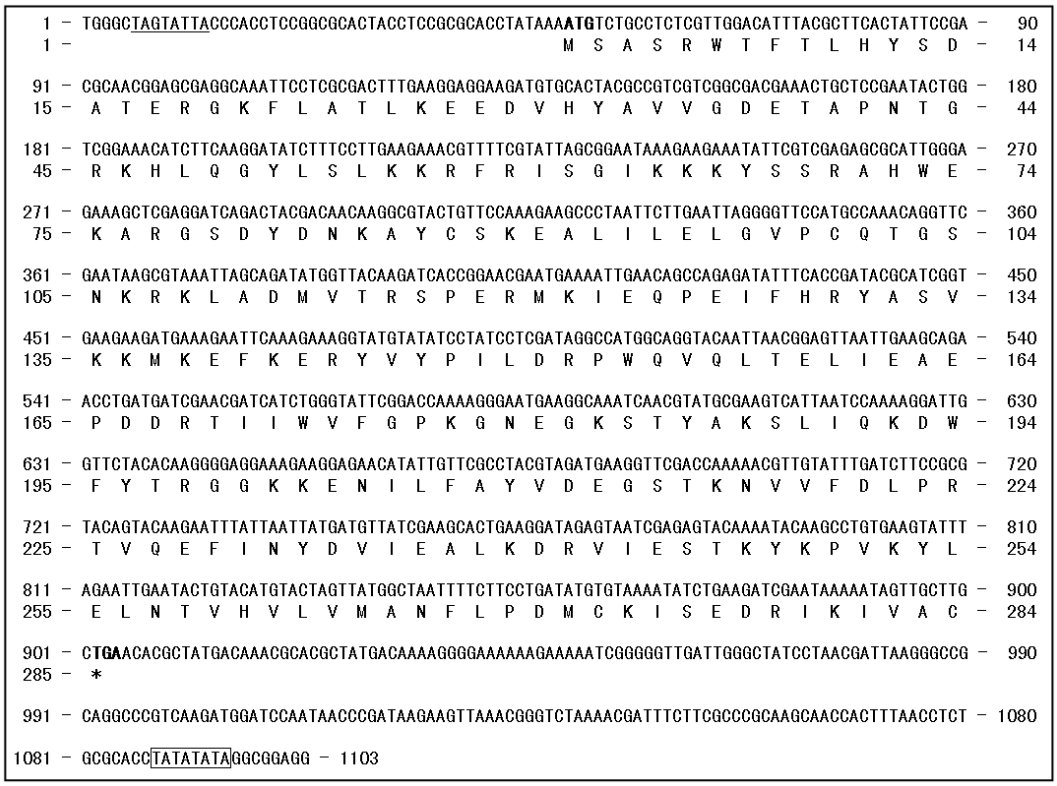



**Fig. 3-4a. Nucleotide and putative amino acid sequence of BBTV DNA-S4**

The conserved 9 nt loop sequence in the intergenic region is underlined. The TATA box is boxed. The start and stop codons are in bold fonts.

**Fig. 3-4b. Alignment of BBTV DNA-S4 (nt 482 to 532) and DNA-S3 (nt 482 to 529) showing the nucleotide and amino acid differences as highlighted by boxes.**

potential protein of 284 aa. The putative aa sequence contained the motif GNEGKS that is conserved amongst BBTV Rep-encoding components. The intergenic region of DNA-S4 contained the stem-loop sequence, CGGAGGTGG**GCTAGTATTAC**CCACCTCCG (the underlined region is the loop; the bold letters indicates the highly conserved loop sequence TAnTATTAC found in all nanoviruses and geminiviruses). Typical of other BBTV satellite DNAs, a putative TATA box was located 5' of the stem-loop while the major common region (CR-M), conserved in the BBTV integral genome components, was absent. DNA-S4 shared 97.6% nt sequence identity with DNA-S3, with all 27 nt differences located within the ORF (Fig. 3-4b); these differences resulted in 12 aa changes between DNA-S3 and -S4.

Phylogenetic analyses of the nt sequences of DNA-S4, other Rep-encoding cssDNA components of nanoviruses and the DNA-1 molecule associated with the begomovirus, *Tomato yellow leaf curl China virus* (TYLCV), revealed that DNA-S4 branched closest to DNA-S3 and these sequences formed a cluster with DNA-S1 and -Y1 (Fig. 3-5). This cluster of sequences was more closely related to DNA-S2 than to the satellite DNAs of MDV and SCSV. The BBTV DNA-R sequences from various geographical isolates were closely related to each other, and these were more closely related to the DNA-1 component associated with TYLCV than to the satellite DNAs of nanoviruses.

## *Effect of DNA-S4 on BBTV replication*

To investigate the effect of BBTV DNA-S4 on replication of BBTV DNA-R, banana cell suspensions were bombarded with 1.1mers of either (1) BBTV DNA-R and -C as a positive control or (2) BBTV DNA-R, -C and -S4. To

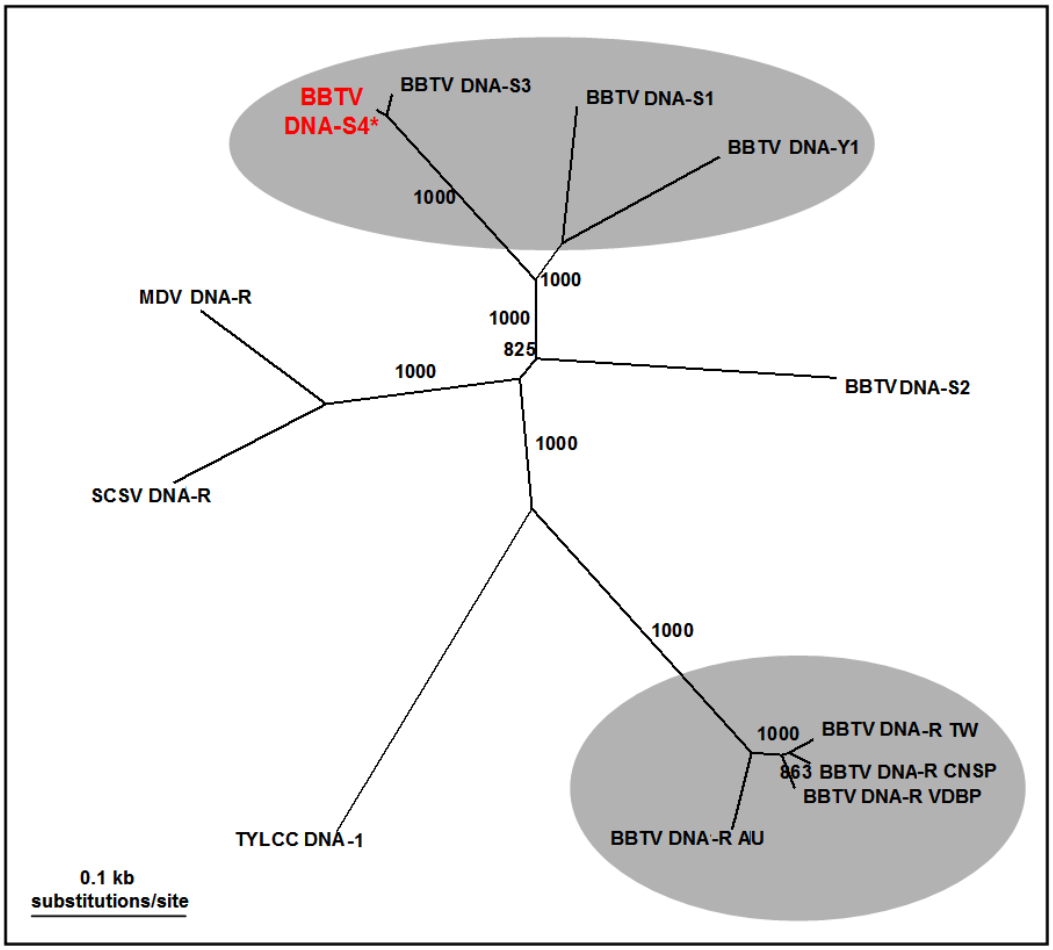



**Fig. 3-5. Phylogenetic analysis of BBTV DNA-S4 and other Rep-encoding sequences**

The nucleotide sequences included in this unrooted neighbor-joining tree include BBTV DNA-R AU (Australian isolate; Accession no. AR010225), CNSP (China isolate; AF239975), VDBP (Vietnam isolate; AF416473) and TW (Taiwan isolate; AF416468); BBTV DNA-S1 (AF216221), -S2 (AF216222), -S3 (AF416471) and -S4 (EU430730); MDV DNA-2 (AB000921); SCSV DNA-2 (NC_003814); and TYLCV DNA-1 (NC_005058). The numbers at the major clades are bootstrap values (1000 replicates).

ensure equal molar amount of DNA were co-bombarded each time, an appropriate amount of the stuffer construct p6.3-NPT-35S-GFP was included when necessary. Three samples from each experimental group were collected on Day 4, 8, 12, 16 and 20 post-bombardment. Untransformed cell suspensions were also collected as negative controls. Total DNA was extracted from samples, Southern blotted and hybridised with a DNA-R specific probe. Replication was assessed qualitatively by presence of different conformational forms of BBTV genomic DNA including open circular, linear and supercoiled, in addition to multimeric intermediates that result from rolling circle replication. Identities of DNA conformations were referenced against molecular weight markers and from previous studies (Horser *et al.*, 2001a). Replication was assessed quantitatively using densitometry readings based on supercoiled, replicative episomal forms of DNA-R.

When 1.1mers of BBTV DNA-R and -C were co-bombarded into embryogenic cells as controls, BBTV-R specific bands were detected on Day 4, 8, 12, 16 and 20 post-bombardment (Fig. 3-6a) indicating that replication of this component had occurred as expected. Although various conformations of viral DNA were observed, supercoiled DNA was the most abundant conformation in each sample. When signal intensities of supercoiled forms of DNA in each experiment were quantitated by densitometry and analysed statistically (Fig. 3-6b), replication of BBTV was found to be highest at 12 days post-bombardment.

When embryogenic cell suspensions were co-bombarded with 1.1mers of BBTV DNA-R, -C and -S4, BBTV-R specific bands were also detected on Day 4, 8, 12, 16 and 20 post-bombardment (Fig. 3-6a). When signal intensities of

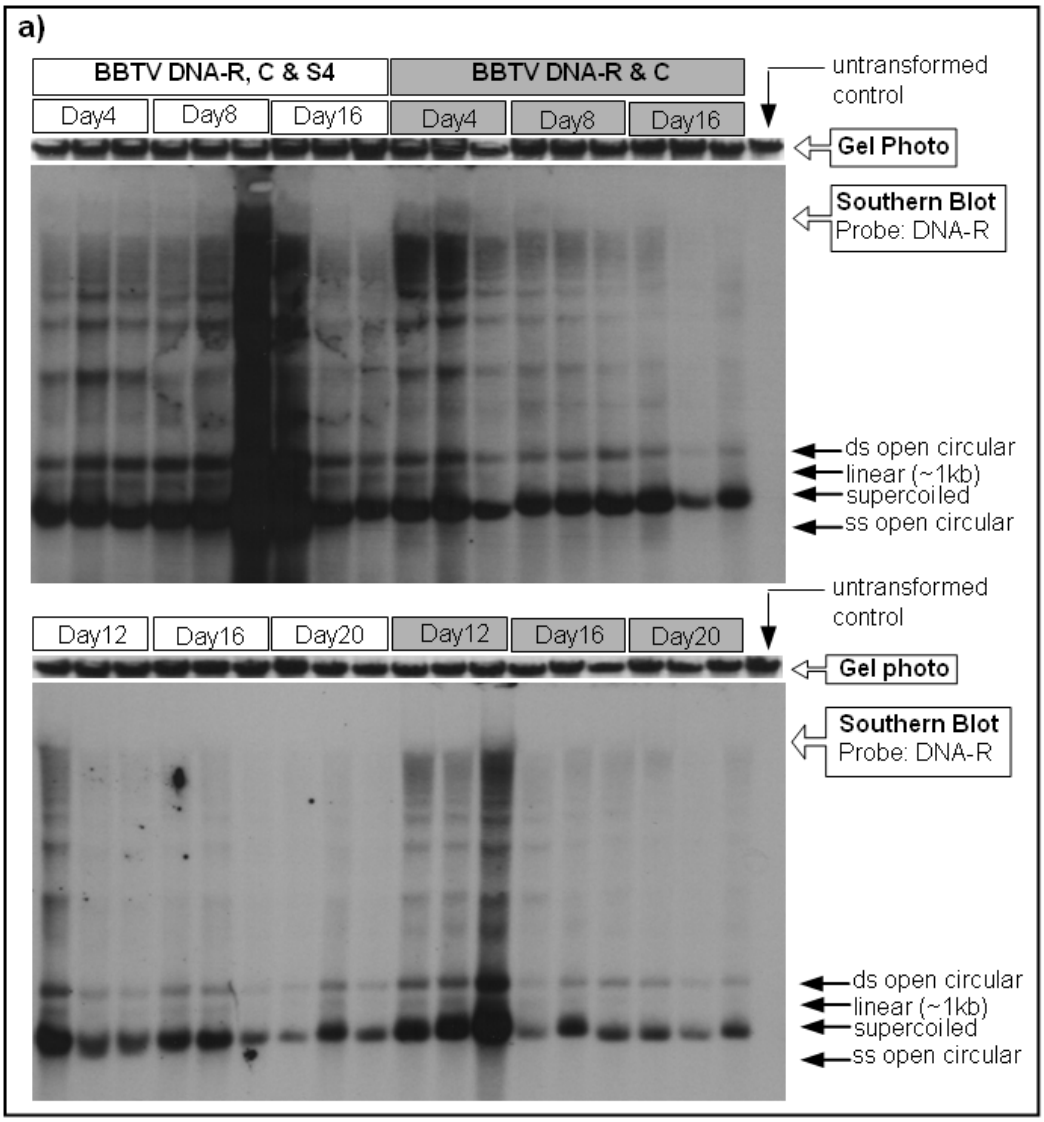



**Fig. 3-6. Replication of BBTV DNA-R in banana embryogenic cell suspensions.** Plasmid clones of 1.1mers of BBTV DNA-R, -C and -S4 were co-bombarded into banana embryogenic cell suspensions.

**(a)** Replication of BBTV DNA-R was examined on Day 4, 8, 12, 16 and 20 post-bombardment by Southern blots using probes specific to BBTV DNA-R. Three or Six replicates are shown for each time point. Both blots were exposed to X-ray film for 1 hour. As indicated on the gel photos, equal amounts of DNA were loaded into each well.

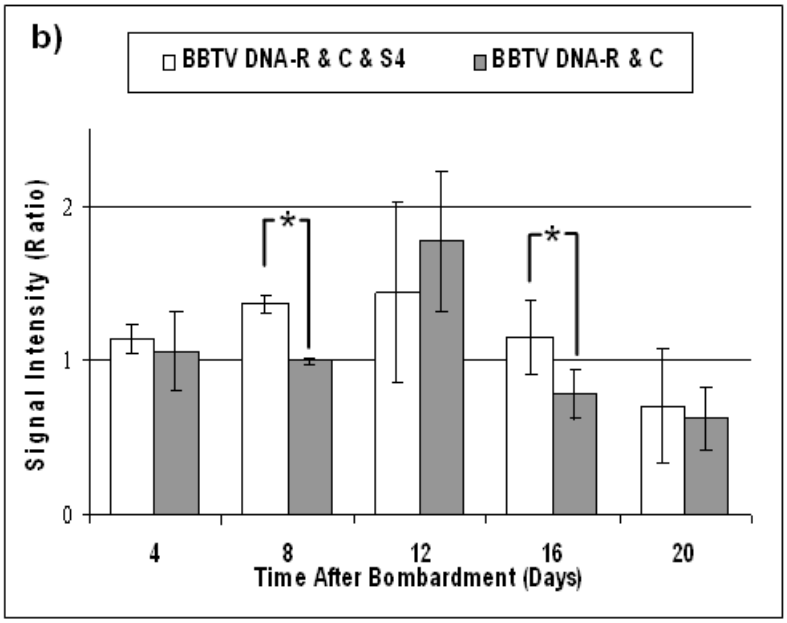



**Fig. 3-6. Replication of BBTV DNA-R in banana embryogenic cell suspensions**

**(b)** Southern signals for supercoiled DNA-R were quantified. The means of signal intensities at each time point are represented as rectangles. The * signs indicate the $P \leq 0.05$ for the rectangle pair. The error bars indicated the 95% confidence intervals. The line across the graph demarcates a ratio of 1, which was the mean intensity of three samples that were bombarded with the plasmid clones of 1.1mers of DNA-R and -C, collected on Day 8 and hybridised with probes specific to DNA-R. The ratio of 0 was the Southern signal intensity of the untransformed control sample.

supercoiled forms of DNA in each experiment were quantitated by densitometry and analysed statistically (Fig. 3-6b), presence of DNA-S4 was shown to significantly enhance replication of DNA-R on Day 8 and 16 post-bombardment, respectively. Although enhancement was also observed at Day 4 and 20, and a slight suppression of replication was observed on Day 12 (Fig. 3-6b), the results were not statistically different.

### *Comparison of the Replicative Capabilities of DNA-S4 and DNA-S1*

Banana cell suspensions were bombarded with 1.1mers of either (1) BBTV DNA-R, -C and -S1, (2) BBTV DNA-R, -C and -S4 or (3) BBTV DNA-R, -C and stuffer construct. Cells were collected on Day 4, 8, 12, 16 and 20 post-bombardment and total DNA was extracted, Southern blotted and initially hybridised with a DIG-ORF-R specific probe. As expected, replication of DNA-R was observed in control cells bombarded with 1.1mers of BBTV DNA-R and -C (Fig. 3-7a). Consistent with the results of Horser *et al* (2001a), the presence of DNA-S1 appeared to weakly suppress replication of DNA-R (Fig. 3-7a). Based on qualitative analysis only, presence of DNA-S4 again appeared to weakly enhance replication of DNA-R (Fig. 3-7a). Blots were then stripped and hybridised with an equal mixture of probes specific for DIG-ORF-S1 and DIG-ORF-S4 (Fig. 3-7b). The specificity and concentration of the probes was verified by dot-blot hybridisation (Fig. 3-7c); based on equal signal strengths to the same amount of target DNA, it was possible to compare signal strengths between experiments. The results obtained using the mixture of DIG-ORF-S1/DIG-ORF-S4 as a probe indicated that DNA-S4 accumulated in cells to considerably higher levels than did DNA-S1 (Fig. 3-7b).

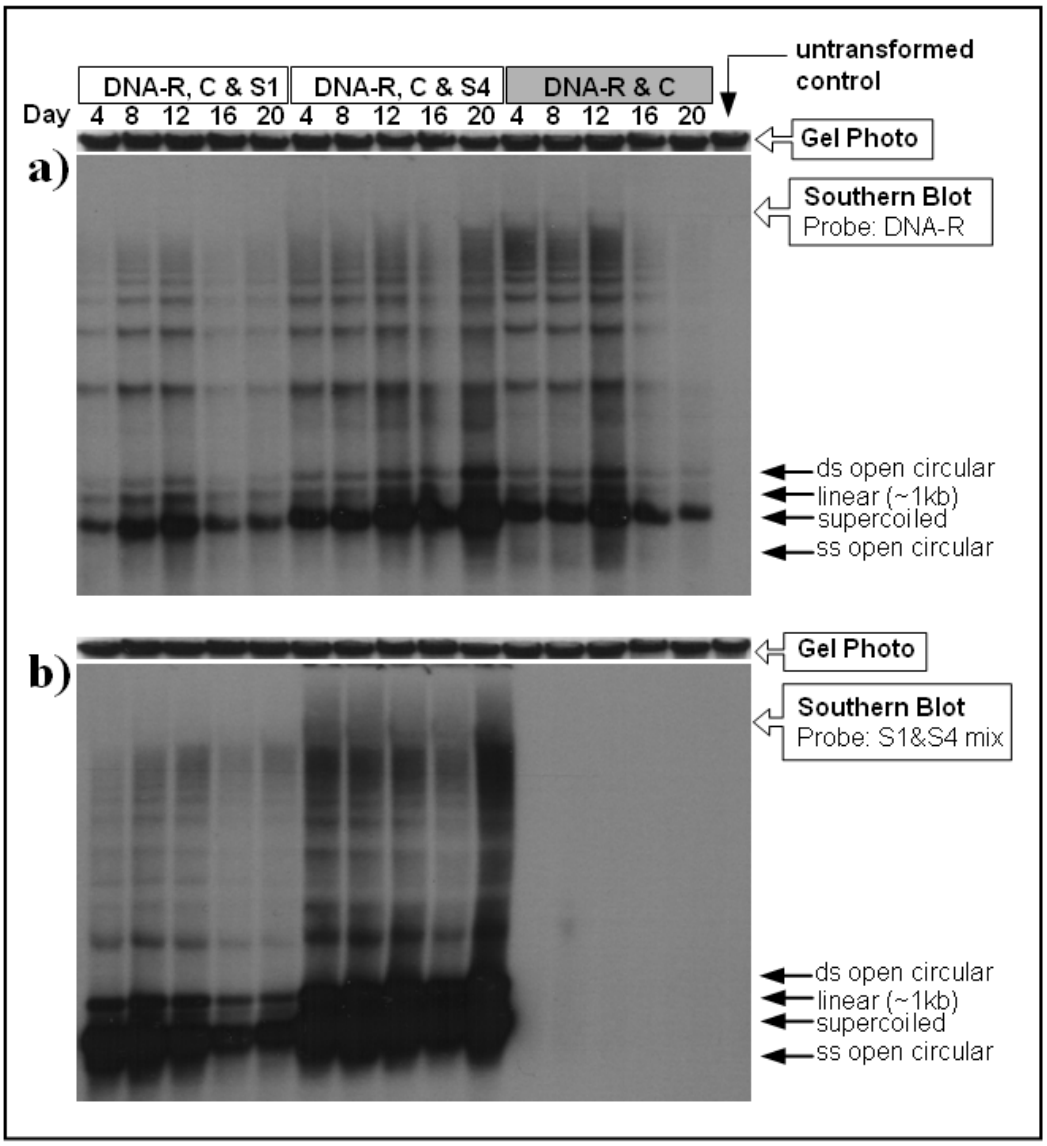



**Fig. 3-7. Replication of BBTV DNA-R, -S1 and -S4 in banana embryogenic cell suspensions**

Plasmid clones of the 1.1mers of BBTV DNA-R, -C, and -S1 or -S4 were bombarded into banana embryogenic cell suspensions. The replication of DNA-R, -S1 and -S4 was examined on Day 4, 8, 12, 16 and 20 post-bombardment by Southern blots using probes specific to **(a)** DNA-R or **(b)** DNA-S1 and -S4. Both blots were exposed to X-ray films for 1 hour. As indicated on the gel photo, equal amounts of DNA were loaded into each well.

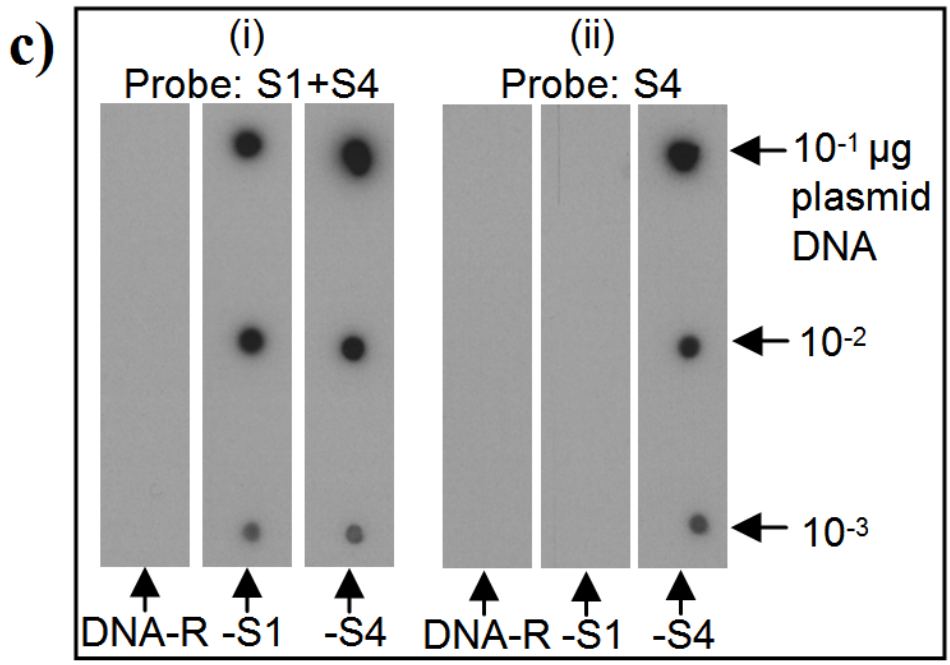



**Fig. 3-7. Replication of BBTV DNA-R, -S1 and -S4 in banana embryogenic cell suspensions**

**(c) Dot blot analysis of DIG-labelled probes.** $10^{-1}$, $10^{-2}$ and $10^{-3}$ µg plasmid clones of the 1.1mers of BBTV DNA-R, -S1 and -S4 were denatured, dotted onto membranes, and hybridised with probes specific to the ORFs of (i) DNA-S1 and -S4 or (ii) DNA-S4 only. The blots were exposed to X-ray film for 2 sec.

# Discussion and conclusions

In this study, we extended the preliminary investigations of Horser *et al.* (2001a) into the effect of the Rep-encoding DNA-S1 on replication of BBTV to assess its potential as a possible transgene for PDR against BBTV. Pathogen-derived resistance (PDR) is a strategy where plants are stably transformed with part of a viral genome (Sanford and Johnston, 1985). In many cases, a proportion of the transformed plants can show resistance against the virus from which the transgene was derived. Although PDR has been successfully used to generate resistance against a large number of RNA viruses, there are limited examples of success against DNA viruses. PDR has been used with moderate success against the cssDNA begomoviruses (Vanderschuren *et al.,* 2007); in many cases, a full-length or truncated, wild-type or mutated Rep ORF has been used as the transgene. PDR against nanoviruses, such as BBTV however, has not been studied. Based on similarities in the replication of geminiviruses and nanoviruses, PDR strategies can potentially be used to control nanoviruses.

To determine the effect of DNA-S1 on replication of BBTV, 1.1mers of BBTV DNA-R, -C and -S1 were bombarded into banana embryogenic cell suspensions. DNA-R encodes the "master" Rep that can initiate replication of integral BBTV genome components (Horser *et al.,* 2001a). DNA-C was included in all bombardments because it encodes the Rb-binding protein, Clink, which creates a cellular environment that allows viral replication possibly by switching the cells into S-phase (Horser *et al.,* 2001a; Wanitchakorn *et al.,* 2000). Southern analyses indicated that BBTV DNA-S1 suppressed the replication of both DNA-R and DNA-S1 significantly on Day 8 and 16 post-bombardment, but had no

significant effect on DNA-R replication on Day 4 post-bombardment. Results were consistent with those obtained previously by Horser *et al* (2001a). The suppressive effect of DNA-S1 on replication of DNA-R is most likely due to competition for cellular replication resources.

Based on the results showing suppression of BBTV-R in the presence of BBTV-S1 1.1mers, use of BBTV-S1 was investigated as a possible transgene to generate PDR against BBTV. As the first stage in this strategy, banana embryogenic cell suspensions were bombarded with pBT1.1-S1 and transgenic plantlets were regenerated. Southern analysis and PCR showed that, in seven banana lines, the transgene was integrated stably into the plant genome. No replicative products of DNA-S1 were detected in leaf tissues. This was not unexpected, as Hermann *et al* (2001) reported that the S1 promoter was active only in vascular tissues of transgenic plants. While none of the transgenic plantlets showed any phenotypic abnormalities, because replication of DNA-S1 was not detected and expression of DNA-S1 ORF was presumably low or absent, we could not determine whether DNA-S1 could induce disease symptoms or had any other effect on transgenic plants. Due to quarantine restrictions, these DNA-S1 transgenic plants were not able to be challenged with BBTV.

In an attempt to increase the suppressive effect of DNA-S1 on BBTV replication, we investigated the effect of over-expression of DNA-S1 ORF on replication of BBTV. The plasmid pUbi-S1.ORF-nos was constructed in which the *ubi*1 promoter controlled expression of the Rep ORF of DNA-S1. Hermann *et al* (2001) showed that the *ubi*1 promoter was > 100 fold stronger than the promoter of DNA-S1 in banana embryogenic cell suspensions. The plasmid

pUbi-S1.ORF-nos was co-bombarded with 1.1mers of DNA-R and -C into banana embryogenic cell suspensions. In contrast to results obtained using 1.1mers of DNA-S1, over-expression of the DNA-S1 ORF significantly enhanced replication of DNA-R on Day 8 and 16 post-bombardment, but did not significantly affect the replication of DNA-R replication on Day 4.

The ~30 amino acid residues near the N-terminus of Reps can only recognise specific iteron sequences, bordering the stem-loop structure in viral genomic DNA, to initiate replication (Timchenko *et al.,* 2000; Horser, 2000). Horser *et al* (2001a) has previously shown that DNA-S1 cannot initiate replication of integral BBTV DNA components including DNA-S, -C and -R. Therefore, observed enhancement of replication by the DNA-S1 Rep in this study was unexpected. It is possible that the Rep of DNA-S1 could assist DNA-R replication indirectly. For example, the ORF of DNA-S1 has been suggested to enhance S1 promoter activity by providing *cis*-regulatory elements to the promoter (Hermann *et al.,* 2001). The same regulatory elements could also enhance the promoter activity of DNA-R *in trans*. Although 1.1mers of DNA-S1 used in the previous study also encoded the ORF of DNA-S1, the replicative ability of the DNA-S1 1.1mer, that may compete with DNA-R for resources required for replication, could potentially counteract enhancement of replication by the ORF of DNA-S1.

In addition, we wished to determine if the effects of DNA-S1 on BBTV replication also occurred with other BBTV satellite DNAs. A satellite DNA, designated BBTV DNA-S4, was isolated from a BBTV-infected banana plant from Vietnam. Interestingly, Bell *et al* (2002) had isolated another satellite DNA, named BBTV DNA-S3, from the same plant. The fact that BBTV DNA-S4 and

-S3 shared 97.6% nt sequence identity and had identical UTRs suggested that DNA-S3 was a sequence variant of DNA-S4. All 12 aa differences in DNA-S4 were located in the Rep-encoding ORF but were outside the conserved rolling circle replication (RCR) domain (Ilyina and Koonin, 1992) and ATPase domain (Gorbalenya *et al.,* 1989; Koonin, 1993); as such, they were unlikely to affect Rep functions.

Rep-mediated initiation of replication of BBTV genomic and satellite DNAs is a sequence-specific process. To initiate replication, the first ~ 30 aa of the Rep protein, consisting of RCR motif 1, are believed to bind to virus-specific iterated sequences (iterons) bordering the stem-loop structure in the untranslatable regions (UTR) of the DNA component (Timchenko *et al.,* 2000). Since the UTRs of DNA-S4 and -S3 were identical, the Rep encoded by DNA-S4 would be expected to initiate replication of both itself and DNA-S3, but not DNA-R.

BBTV DNA-S4 replicated at very high levels in the presence of DNA-R. Further, DNA-S4 was shown to replicate more efficiently than DNA-S1. In contrast to the results of Horser *et al* (2001a) and our results on DNA-S1, we found that DNA-S4 did not suppress, but significantly enhanced, replication of DNA-R in banana cells on Day 8 and 16 post-bombardment. It is not clear why DNA-S4 did not have any significant effect on replication of BBTV DNA-R on Day 4, 12 and 20. On Day 12, the explanation may be due to biological variation or sampling error. Although replication of both BBTV integral genome and satellite DNAs have all been observed on Day 4 and 20 in this study, replication rate was usually not at its highest. It is possible that either the low level of replication of the satellite DNAs or low Rep expression by satellite DNAs were

not sufficient to affect the replication of BBTV DNA-R in cells on Day 4 and 20 post-bombardment significantly. It is also possible that it may take longer than four days for transgenes to be expressed at their optimal level in bombarded cells. Further, on Day 20, it is possible that the transgenes that have not integrated into the genome of bombarded cells may have degraded.

Based on the very high levels of satellite DNAs in the presence of DNA-R and the isolation of these satellite components from plants before DNA-R, it has been suggested that BBTV satellite DNAs occur in higher concentrations than integral genomic components (Horser *et al.,* 2001a). As such, presence of satellite DNAs could suppress replication of DNA-R, as shown for DNA-S1, by competing for replication essentials. The observation that both pUbi-S1.ORF-nos and DNA-S4 enhanced replication of DNA-R in this study was, therefore, unexpected. Although the reason for this has not been determined, it could possibly be related to differing promoter activity levles of DNA-S1 and -S4 resulting in different levels of Rep expression. The S4 promoter may be stronger than S1 promoter and could have expressed more Rep proteins to thus enhancing DNA-R replication. It is also possible that the Rep proteins of DNA-R/S1 and DNA-R/S4 interact in different ways due to differences in DNA-S1 and -S4 sequences, thus affecting BBTV replication.

In conclusion, the observation that DNA-S4 enhanced replication of DNA-R in transiently transformed banana cells precludes transgenic expression of this satellite DNA, or the phylogenetically closely-related DNA-S3, as a possible control strategy for BBTV. In contrast, transgenic expression of DNA-S1 offers greater potential as a control strategy since this component has been shown to

suppress replication of DNA-R.

# CHAPTER 4

## THE REPLICATION OF *BANANA BUNCHY TOP VIRUS* IS SUPPRESSED BY OVER-ABUNDANT REP EXPRESSION

# Abstract

The genome of *Banana bunchy top virus* (BBTV) contains at least six circular, single stranded (css) DNA components, known as DNA-R, -U3, -S, -M, -C and -N. Each of these DNA components is ~ 1 kb in size, and each encodes one open reading frame (ORF) except for DNA-R. DNA-R has two ORFs. The large ORF of DNA-R encodes a replication initiation protein (Rep). The small U5 ORF is located within the large ORF. In this study, we found that when the Rep was over-expressed but the U5 ORF was untranslatable, excess Rep transcripts abolished replication of the BBTV genome. When the Rep and U5 genes were both over-expressed, replication of BBTV was enhanced. When the U5 was over-expressed alone without the Rep, replication of BBTV was weakly suppressed. Results of this study suggest therefore, that U5 may encode a suppressor of post-transcriptional gene silencing (PTGS). Over-expression of the BBTV Rep gene with an untranslatable internal U5 ORF could have the potential to confer plants with genetically engineered resistance against BBTV by triggering PTGS of the viral Rep gene.

# Introduction

# Introduction

*Banana bunchy top virus* (BBTV) is one of the most severe pathogens of banana. The integral genome of BBTV consists of six circular, single-stranded (css) DNA components, namely BBTV DNA-R, -U3, -S, -M, -C and -N, that are found consistently with all isolates of the virus (Burns *et al.,* 1994). All DNA components are ~ 1 kb in size, and are replicated by a rolling circle mechanism (Burns *et al.,* 1995; Hafner *et al.,* 1997a).

BBTV DNA-R consists of two open reading frames (ORF), one internal to the other (Beetham *et al.,* 1997). The large ORF of DNA-R encodes a replication initiation protein (Rep) while the small, internal ORF encodes a protein (designated U5) of unknown function (Hafner *et al.,* 1997b; Beetham *et al.,* 1997). The Rep encoded by DNA-R is considered to be the BBTV "master" Rep (M-Rep) because it can initiate replication of DNA-R as well as other integral genome components (Horser *et al.,* 2001a). The other BBTV DNA components each encode a single ORF (Burns *et al.,* 1995). The function of DNA-U3 is unknown, while DNA-S, -M, -C and -N encode the coat protein, movement protein, cell-cycle link (Clink) protein and nuclear shuttle protein, respectively (Wanikchakorn *et al.,* 1997; 2000).

Horser (2000) used 1.1mers (i.e. greater-than-unit-length artificial constructs that contain two intergenic regions of the viral genome) to examine the effect of two different mutations in the Rep gene on replication of BBTV using 1.1mers.

Construct "Rep-" comprised a 1.1mer of BBTV DNA-R, where the U5 ORF was mutated such that it was untranslatable but the Rep gene was translatable with no change to its amino acid sequence; while construct "IntORF" comprised a 1.1mer of BBTV DNA-R where the Rep gene was rendered untranslatable without altering the amino acid sequence of the internal ORF. In banana embryogenic cell suspensions, self-replication was shown to be initiated by "Rep-", but not by "IntORF"; the self-replication of "Rep-" was less than that of the native DNA-R (Horser, 2000). When constructs "Rep-" and "IntORF" were co-bombarded into cells, thus providing both genes in a translatable form but *in trans* instead of *in cis*, self-replication of "Rep-" was further suppressed (Horser, 2000). Presence of two replicative components, "Rep-" and "IntORF", may suppress replication by competing for limited cellular resources required for replication.

To further understand the roles of the gene products of the two ORFs on DNA-R, the effect of over-expression of the two ORFs on BBTV replication using the strong, constitutive maize polyubiquitin 1 (*ubi*1) promoter was investigated.

# Materials and methods

# Materials and methods

## *Generation of constructs*

### pBT1.1-R and pBT1.1-C

Constructs comprised 1.1mers of BBTV DNA-R (GenBank Accession No. NC_003479) and -C (GenBank Accession No, NC_003477), respectively, ligated into pGEM-T (Promega) (Fig. 4-1), that were generously provided by Dr. Cathryn Horser (Horser *et al.,* 2001a). Plasmids pBT1.1-R and pBT1.1-C had been designated previously as pBT1.1-1 and pBT1.1-5, respectively (Horser *et al.,* 2001a).

### pUbi-R.ORF-nos, pUbi-RepOnly-nos and pUbi-IntOnly-nos

These constructs contained the maize polyubiquitin 1 (*ubi*1) promoter controlling the expression of (1) both the M-Rep and U5 proteins encoded by DNA-R (i.e. both the native DNA-R ORFs) (2) only the M-Rep of DNA-R and (3) only the internal U5 ORF of DNA-R, respectively (Fig. 4-1). The major M-Rep ORF (nt 129-989 of BBTV DNA-R Australian isolate) was amplified from pBT1.1-R using primers Bam_ORF1_F and Sac_ORF1_R (Table 4-1) in a PCR, using pBT1.1-R or "Rep-"as the templates. The "Rep-" was a plasmid clone of BBTV DNA-R 1.1mer from a previous study (Horser, 2000) in which expression of U5 ORF was abolished by point mutations. The U5 ORF (nt 403-531 of BBTV DNA-R Australian isolate) was amplified using primers, Bam_Int_F and Sac_Int_R and pBT1.1-R, as the template.

The PCR mixtures consisted of 10 pmol of each primer, 200 μM dNTPs, 1

U Expand DNA polymerase (Roche), and 0.1 µg of template DNA (pBT1.1-R or "Rep-" 1.1mer dissolved in sterilised $H_2O$) in 1 x Expand PCR Buffer 1 (Roche).

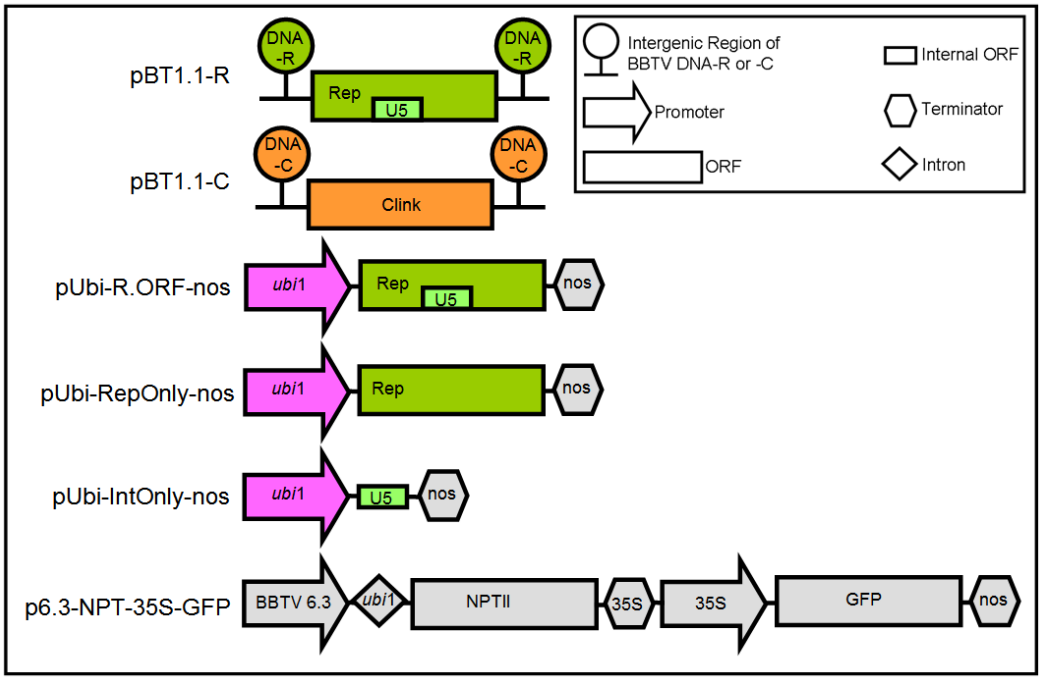



**Fig. 4-1. Maps of constructs.**

The *ubi*1 promoter and intron are the promoter and intron of maize polyubiquitin 1 gene. The *nos* terminator is the terminator sequence of a nopaline synthase. The BBTV 6.3 promoter was derived from BBTV DNA-N (Dugdale *et al.*, 1998). The NPTII gene encodes the neomycin phosphotransferase II. The CaMV 35S promoter and terminator are the promoter and terminator sequences from *Cauliflower mosaic virus* (CaMV). The GFP gene encodes the green fluorescent protein from *Aequorea victoria*.

**Table 4-1. List of primers.**

| Name | Sequence* (5' to 3') | Location (nt) |
|---|---|---|
| ORF1F | ATGGCGCGATATGTGGTATGCT | 129-149 of DNA-R |
| ORF1R | TCAGCAAGAAACCAACTTTAT | 989-968 of DNA-R |
| Bam_ORF1_F | **GGATCC**ATGGCGCGATATGTGGTATGCT | 129-149 of DNA-R |
| Sac_ORF1_R | **GAGCTC**TCAGCAAGAAACCAACTTTAT | 989-968 of DNA-R |
| Bam_Int_F | **GGATCC**ATGATAATTTATTTGATGTCATAC | 403-426 of DNA-R |
| Sac_Int_R | **GAGCTC**AGTAGAGACGAACATGAGACATAT | 531-508 of DNA-R |
| BT5_240F | ATGGAGTTCTGGGAATCGTCTGCCATG | 240-266 of DNA-C |
| BT5_725R | TTAGAGTAATGTTACATCATAGTCTGA | 725-699 of DNA-C |
| 1_R_B2B | GAAGTTCTCCTAGCTATTCATCG | 464-442 of DNA-R |
| 1_F_B2B | GTGCTTGGACATCAGAGGTG | 470-489 of DNA-R |
| 5_R_B2B | GATCTCCATAAACAAGAATCC | 139-119 of DNA-C |
| 5_F_B2B | GATGCCCTTGCCGGAGTGA | 145-163 of DNA-C |

** Sequences in bold are restriction sites.*

Reaction mixtures were heated at 92 °C for 2 min, followed by 35 cycles of 92 °C for 30 sec, 50 °C for 30 sec and 68 °C for 90 sec, followed by 1 cycle of 72 °C for 10 min. PCR products were ligated into pGEM-T (Promega) at 14 °C for 16 hours using 2 U of T4 DNA ligase (Roche). The ligation was transformed into *Escherichia coli* DH5α using a 2 min/42 °C heat shock. Plasmids were extracted from selected clones, digested with *Bam*HI and *Sac*I enzymes, electrophoresed through 1% agarose gels in TAE buffer, pH 7.8, and stained with ethidium bromide. Amplicons of ~ 850 bp (the Rep ORF) and ~ 130 bp (the U5 ORF) were then excised and purified from the gel using a High Pure Gel Extraction Kit (Roche). Resulting fragments were inserted into *Bam*HI/*Sac*I sites located between the *ubi*1 promoter and nopaline synthase (*nos*) terminator, in the plasmid pGEM-*ubi*-*nos.*

**p6.3-NPT-35S-GFP**

This construct (Fig. 4-1) was available from previous work and was generously provided by Mr. Matthew Webb (QUT). It comprised the BBTV 6.3 promoter-*ubi*1 intron-NPTII-CaMV 35S terminator together with the CaMV 35S promoter-GFP-*nos* terminator. This plasmid was used as a "stuffer" construct to ensure equal molar amounts of DNA were used in experiments. Further, the plasmid served as a reporter gene (GFP) following microprojectile bombardment.

## *Sequence analysis*

All constructs were purified using a BRESA-pure MAXi-prep Plasmid Purification kit (Geneworks). Constructs were sequenced using an automatic sequencer and Big Dye Termination Cycle Sequencing Ready Reaction V3.1 (PE Applied Biosystems). Primers used for sequencing included specific primers listed in Table 4-1 and M13 universal sequencing primers (US Biochemical).

## *Transformation by microprojectile bombardment*

Banana "Lady Finger" (*Musa* spp. AAB group) somatic embryogenic cell suspension cultures were prepared and maintained by Jennifer Kleidon (QUT) as described by Khanna *et al.* (2004). Somatic embryos were harvested and approximately 0.1 g of condensed cell suspension was plated onto a filter paper, then placed on solid Bluggoe Low culture media (Dheda *et al.,* 1991). Each plate was bombarded with 1 μg of plasmid of the constructs using a particle inflow gun and gold microcarriers (BioRad) essentially as described by Dugdale *et al.* (1998).

Banana embryogenic cell suspensions were bombarded with combinations of plasmid constructs. On Day 4, 8 or 16 post-bombardment, transformation efficiency was monitored by observing the GFP expression in cells using a Leica MZ12 stereo microscope with GFP-Plus fluorescence module and green barrier filter (BGG22, Chroma Technology). Cell samples were also collected on these days. Cells from different plates were stored in Eppendorf tubes at -80 °C prior to analysis.

## *Nucleic acid extraction*

Total nucleic acids were extracted from cells and dissolved in TE (pH 8) essentially as described by Stewart and Via (1993). RNA was removed by RNase A (Roche) digestion, and the concentration of DNA was quantified by spectrophotometry (Sambrook and Russell, 2000).

## *Generation of digoxigenin (DIG)-labeled probes*

DIG-ORF-R and DIG-ORF-C were DIG-labeled DNA probes for the specific detection of the ORFs of BBTV DNA-R and -C, respectively.

DIG-ORF-R was amplified from pBT1.1-R using primers ORF1F and ORF1R, while DIG-ORF-C was amplified from pBT1.1-C using primers BT5_240F and BT5_725R. PCRs were done as described previously. The PCR products were electrophoresed through 1% agarose gels in TAE buffer, pH 7.8, and stained with ethidium bromide. PCR products of ~ 850 bp (DIG-ORF-R) and ~ 500 bp (DIG-ORF-C) were excised and purified from the gel using a High Pure Gel Extraction Kit (Roche). Products were used as templates for the second round of PCR, in which dNTPs were replaced by 5 µl DIG labeling mix (Roche) to incorporate the DIG-label into PCR products. The probes were purified using a QIAquick PCR purification kit and their concentration was quantified by spectrophotometry (Sambrook and Russell, 2000).

### *Analysis of transient transformants*

The replication and accumulation of BBTV DNA-R and DNA-C was studied using Southern analysis. Total nucleic acids were extracted from bombarded cell suspensions and (20 µg) electrophoresed through 1.5 % agarose gels in 1 x TAE buffer (pH 7.8), and stained with ethidium bromide. Total nucleic acid (20 µg) from untransformed banana embryogenic cell suspensions was included as a negative control. Size of DNA fragments on the agarose gels was determined using DIG-labeled molecular marker III (Roche). Nucleic acids were transferred from the agarose gel to positively charged nylon membranes (Roche) by capillary blotting for 16 hours (Southern, 1975). The nylon membranes were baked at 80 °C for 2 hours, pre-hybridised in DIG-Easy Hyb (Roche) at 42 °C for 1-2 hours, hybridised with 250 nmol of DIG-ORF-C in 10 ml of DIG-Easy Hyb at 42 °C for 12-16 hours, washed at high stringency (0.1 x SSC, 0.1 % SDS) at 65 °C prior to development as per the manufacturer's instructions (Roche), and

exposed to X-Ray films (AGFA) for 30 min. Membranes were stripped as per the manufacturer's instruction (Roche), hybridised with 250 nmol ORF-DIG-R, developed and exposed again to X-Ray films for 30 min. The X-ray films were developed by automatic developer (AGFA).

Total DNAs (0.1 μg) extracted from each samples of bombarded cell suspensions were also used as templates for PCR using conditions described previously. Adjacent, outwardly-extending primer pairs (1_F_B2B/1_R_B2B or 5_F_B2B/5_R_B2B primers) were used in the PCR to specifically amplify DNA-R and -C, respectively.

# Results

# Results

Three BBTV DNA-R mutants were constructed; plasmid pUbi-RepOnly-nos contained the *ubi*1 promoter driving Rep expression from DNA-R (with untranslatable U5 ORF), plasmid pUbi-IntOnly-nos contained the *ubi*1 promoter driving expression of the DNA-R internal gene product (U5) (with deleted Rep ORF), while plasmid pUbi-R.ORF-nos contained the *ubi*1 promoter driving the expression of both Rep and the internal U5 gene product. Banana embryogenic cell suspensions were bombarded with various combinations of plasmid constructs, including (1) pBT1.1-R and pBT1.1-C; (2) pBT1.1-R, pBT1.1-C and pUbi-IntOnly-nos; (3) pBT1.1-R, pBT1.1-C and pUbi-RepOnly-nos; (4) pBT1.1-R, pBT1.1-C and pUbi-R.ORF-nos; (5) pBT1.1-C and pUbi-IntOnly-nos; (6) pBT1.1-C and pUbi-RepOnly-nos; (7) pBT1.1-C and pUbi-R.ORF-nos; (8) pBT1.1-R and pUbi-IntOnly-nos; (9) pBT1.1-R and pUbi-RepOnly-nos or (10) pBT1.1-R. To ensure an equimolar amount of DNA was co-bombarded each time, appropriate amounts of the stuffer construct p6.3-NPT-35S-GFP were also included where needed. Cells were collected on Day 4, 8 or 16 post-bombardment. Untransformed cells were also collected as the negative control. Total DNA was extracted from cell samples, Southern blotted, hybridised with a probe specific to DNA-C, stripped and re-hybridised with a DNA-R specific probe. Replication was assessed qualitatively by presence of different conformational forms of BBTV genomic DNA including open circular, linear and supercoiled, in addition to multimeric intermediates occurring from RCR. Identities of DNA conformations were based on reference to molecular weight markers and from previous studies (Horser *et al.,* 2001a).

Replication and re-circularisation of 1.1mers was also assessed by PCR using adjacent, outwardly-extending ("back-to-back") primers specific to DNA-R and -C. These primers were designed such that amplification would only occur if the 1.1mers had been nicked at the stem-loops and re-circularised into monomeric or formed multimeric intermediates.

## *BBTV DNA-C replication was not initiated by pUbi-RepOnly-nos or pUbi-IntOnly-nos*

To determine whether replication of an integral BBTV genome component (DNA-C) could be initiated by either Rep minus the U5 gene product (pUbi-RepOnly-nos) or the U5 gene product alone (pUbi-IntOnly-nos), and to determine the effect of over-expression of native Rep on DNA-C, pBT1.1-C (a plasmid clone of BBTV DNA-C 1.1mer) was co-bombarded into banana embryogenic cell suspensions with various combinations of BBTV gene constructs.

In control cells bombarded with pUbi-R.ORF-nos and DNA-C (pBT1.1-C) and examined on Day 8 post-bombardment, DNA-C specific bands were detected, indicating that successful replication of this component had occurred (Fig. 4-2). In contrast, no BBTV DNA-C specific bands were observed in equivalent DNA from cells that were co-bombarded by 1.1mers of DNA-C with either pUbi-RepOnly-nos or pUbi-IntOnly-nos (Fig. 4-2). The above results were also supported from PCR analyses using "back-to-back" primers (Table 4-2).

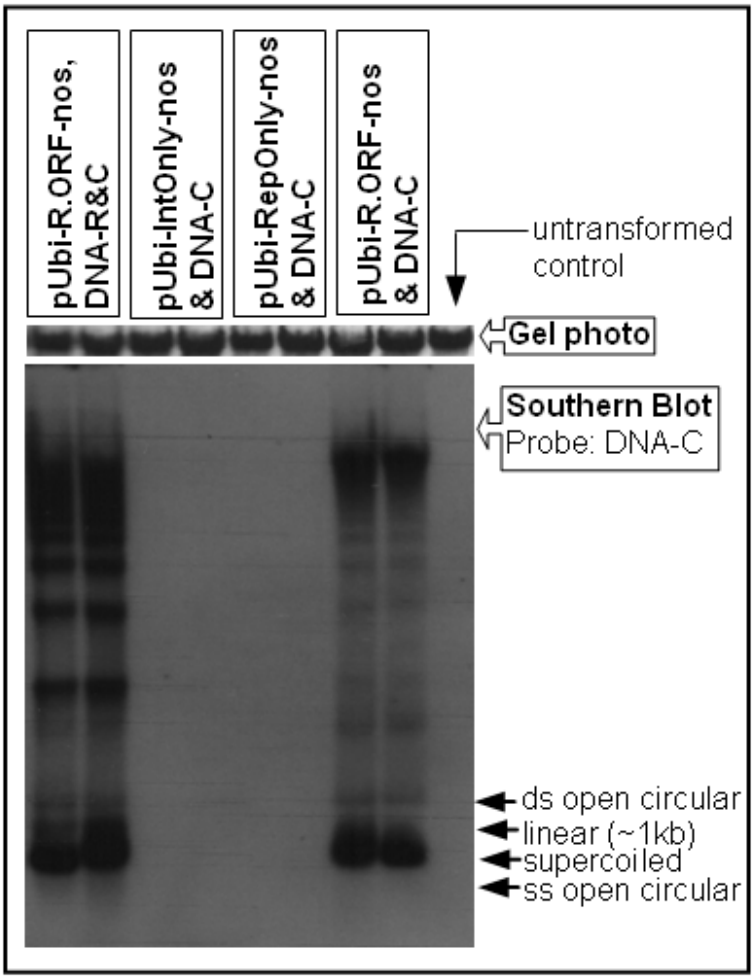



**Fig. 4-2. Replication of BBTV DNA-C *in vivo***

Banana embryogenic cell suspensions were bombarded with various combinations of BBTV gene constructs. The replication of DNA-C in the cells on Day 8 post-bombardment was examined by Southern blots using probes specific to DNA-C. Equal amounts of undigested DNA were loaded in each lane as shown in the gel photo. Two replicates are shown for this time point.

**Table 4-2. Summary of Southern analysis and PCR detection**

| Bombarded Constructs | Southern Analyses | | PCR detection | |
|---|---|---|---|---|
| | DNA-R | DNA-C | DNA-R | DNA-C |
| DNA-R 1.1mer<br>DNA-C 1.1mer | + | + | + | + |
| DNA-R 1.1mer<br>DNA-C 1.1mer<br>pUbi-IntOnly-nos | + | + | + | + |
| DNA-R 1.1mer<br>DNA-C 1.1mer<br>pUbi-RepOnly-nos | - | - | - | - |
| DNA-R 1.1mer<br>DNA-C 1.1mer<br>pUbi-R.ORF-nos | +++ | +++ | +++ | +++ |
| DNA-C 1.1mer<br>pUbi-IntOnl-nos | - | - | - | - |
| DNA-C 1.1mer<br>pUbi-RepOnly-nos | - | - | - | - |
| DNA-C 1.1mer<br>pUbi-R.ORF-nos | - | ++ | - | ++ |

Cells were bombarded with various combinations of BBTV gene constructs, collected on Day 8 post-bombardment, and analysed by Southern blots and PCR. The "-" indicates BBTV DNA-R or -C was not detected, while "+" indicates a weak positive signal, "++" indicates intermediate signal and '+++' indicates strong signal.

pUbi-R.ORF-nos enhanced BBTV replication, while pUbi-RepOnly- nos suppressed BBTV replication

Southern (Fig. 4-2) and PCR analyses (Table 4-2) both showed that pUbi-R.ORF-nos was capable of initiating replication of DNA-C in banana embryogenic cell suspensions. Further, when pUbi-R.ORF-nos was co-bombarded with pBT1.1-R and pBT1.1-C, replication of both DNA-R and -C was significantly enhanced by presence of pUbi-R.ORF-nos on Day 8 post-bombardment (Fig. 4-3; Table 4-2).

When pBT1.1-R and pBT1.1-C were co-bombarded with pUbi-RepOnly-nos into banana embryogenic cell suspensions, replication of both DNA-R and -C was suppressed on Day 4, 8 and 16 post-bombardment (Fig. 4-3 and -4; Table 4-2). Consistent with previous studies by Horser *et al.* (2001a), the control bombardments showed that DNA-R was capable of self-replication and that presence of pBT1.1-C enhanced DNA-R replication. Interestingly, DNA-R replication was always suppressed by pUbi-RepOnly-nos, in the presence or absence of pBT1.1-C (Fig. 4-4). In contrast, when pBT1.1-R and pBT1.1-C were co-bombarded with pUbi-IntOnly-nos into cell suspensions, replication of DNA-R and -C were only weakly suppressed (Fig. 4-3 and -4).

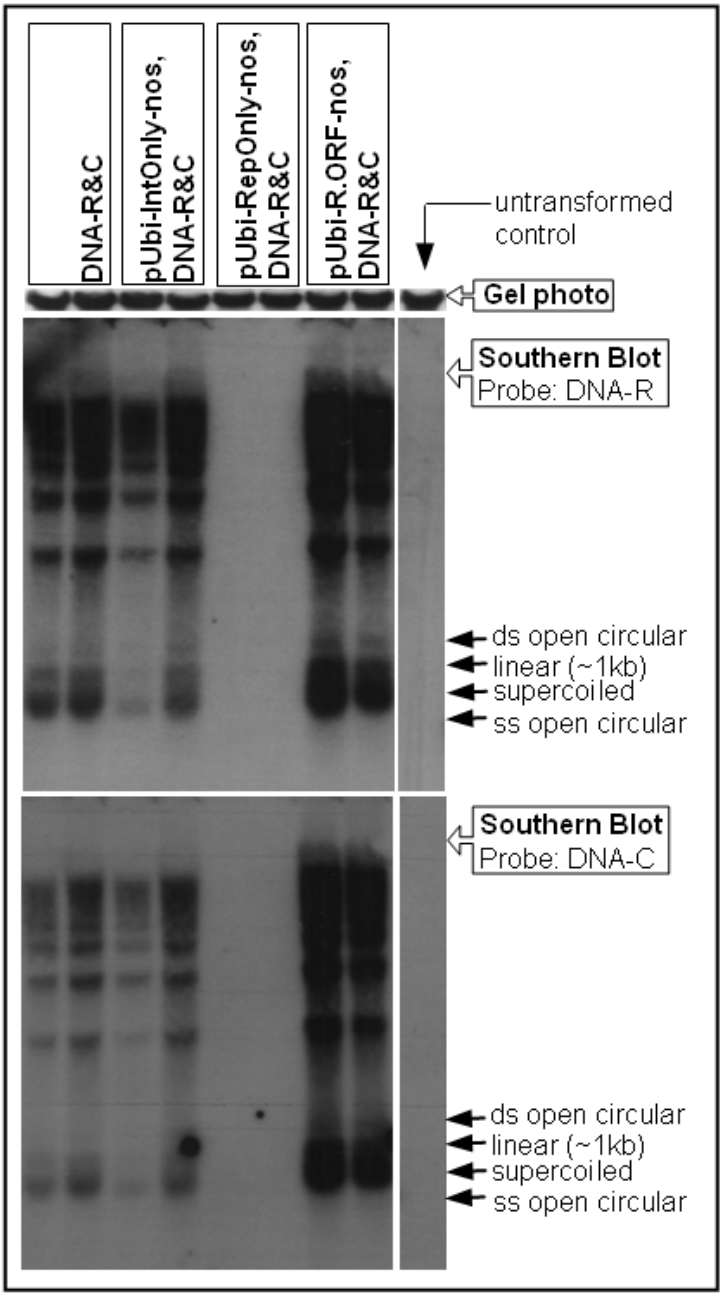



**Fig. 4-3. Replication of BBTV DNA-R or -C *in vivo***

Banana embryogenic cell suspensions were bombarded with various combinations of constructs as indicated. The cells were collected on Day 8 post-bombardment, and the accumulation of BBTV DNA-R or -C in the cells was detected by Southern blots using probes specific to DNA-R. The blot was stripped and re-hybridised with probes specific to DNA-C. Equal amounts of undigested DNA were loaded in each lane as shown. Two replicates are shown for this time point.

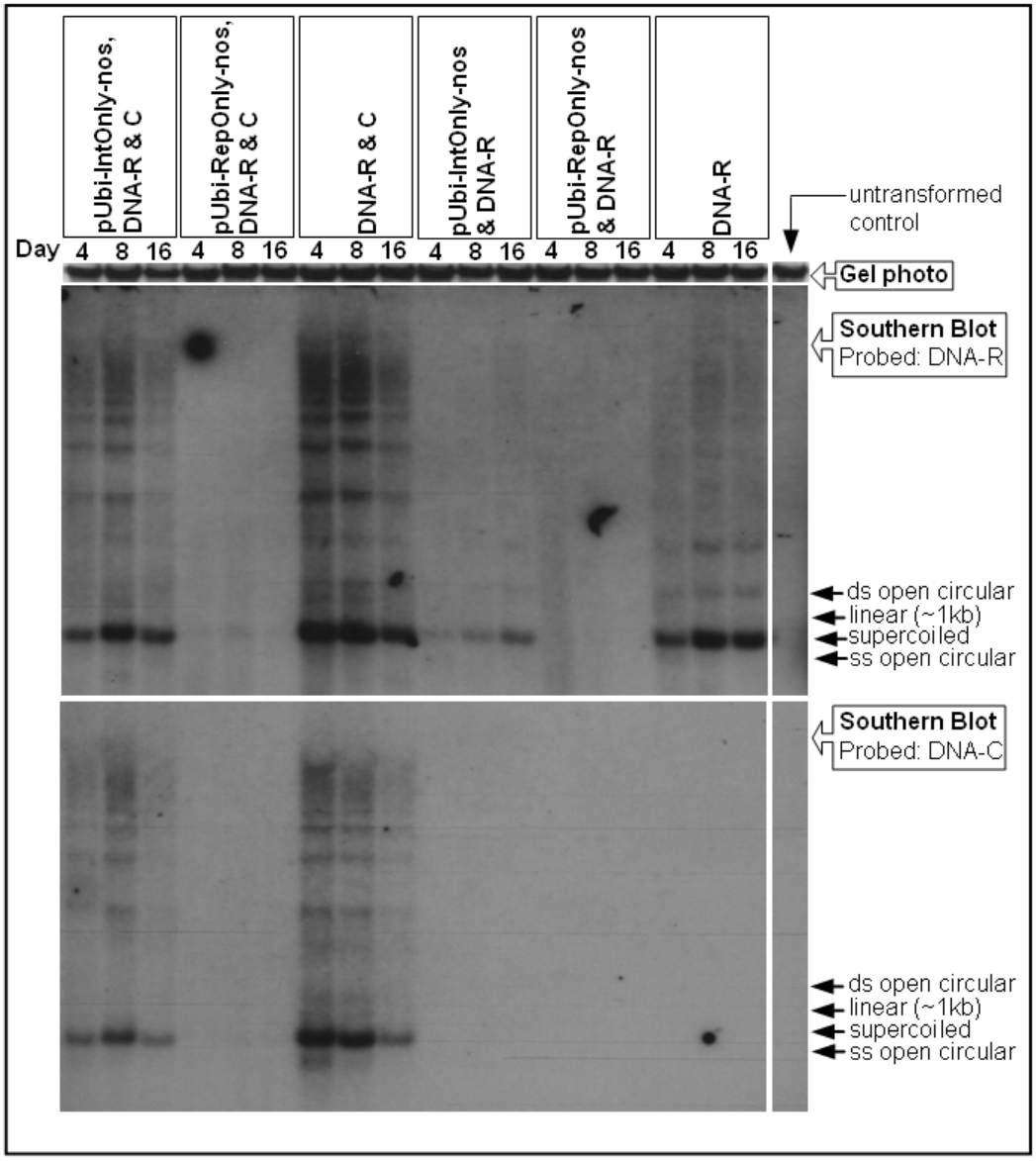



**Fig. 4-4. Replication of BBTV DNA-R or -C *in vivo*.**

Banana embryogenic cell suspensions were bombarded with various combinations of BBTV gene constructs as indicated. The cells were collected on Day 4, 8 or 16 post-bombarded, and the replication of BBTV DNA-R or -C in the cells was analysed by Southern blots using probes specific to DNA-C. The blot was stripped and hybridised again with probes specific to DNA-R.

# Discussion and conclusions

BBTV is a nanovirus belonging to the genus *Babuvirus* of the family *Nanoviridae*. Both nanoviruses and geminiviruses are small viruses with cssDNA genomes where replication is controlled by the viral Rep. The viral Rep genes have been used to genetically engineer plants with resistance to infection by several geminiviruses, including *Tomato yellow leaf curl Sardinia virus*, *Tomato mottle virus*, *African cassava mosaic virus* (ACMV), and *Mungbean yellow mosaic virus-Vigna* (Lucioli *et al.,* 2003; Polston *et al.,* 1998; Hong and Stanley, 1996; Chellappan *et al.,* 2004b; Shivaprasad *et al.,* 2006). However, potential for Rep-mediated resistance against nanoviruses has not yet been evaluated.

The results of this study suggest that pUbi-RepOnly-nos may be a suitable transgene for generating resistance against BBTV. This is based on the observation that pUbi-RepOnly-nos strongly suppressed replication of BBTV 1.1mers in banana embryogenic cells. Plasmid pUbi-RepOnly-nos had the strong *ubi*1 promoter over-expressing the BBTV Rep, whereas the U5 ORF located internal to the Rep ORF has been mutated to be untranslatable. Although the precise mechanism of suppression is unknown, it is possible that over-expression of Rep proteins could inhibit transcription of Rep from DNA-R by specifically bindng to the promoter of Rep in BBTV DNA-R (Brunetti *et al.,* 2001). Alternatively, mRNA transcripts of Rep that were over-expressed by pUbi-RepOnly-nos might trigger post-transcriptional gene silencing (PTGS) of Rep (Szittya *et al.,* 2002).

We also found that plasmid pUbi-RepOnly-nos was unable to initiate replication of DNA-C. This was also possibly due to PTGS of the over-expressed Rep gene, because when expression of BBTV Rep was controlled by a weaker promoter, such as the native promoter of BBTV Rep in DNA-R, BBTV Rep alone could initiate the replication of DNA-C (Horser, 2000).

Transgenes based on pUbi-RepOnly-nos might also confer resistance against not only BBTV, but also other nanoviruses by triggering PTGS of Rep genes. Although it was traditionally believed that PTGS could only occur between genes with > 90% homology in nucleotide sequences, recent studies have shown that lower level of homology may be acceptable (Ritzenthaler, 2005; Chellappan *et al.,* 2004b). For example, the full-length wild-type Rep gene of ACMV has been used to confer PTGS-mediated resistance against *East African cassava mosaic Cameroon virus* and *Sri Lankan cassava virus*, with Rep proteins sharing only 66% and 67% homology nucleotide sequence to the Rep of ACMV, respectively (Chellappan *et al.,* 2004b). Resistance against more distantly related viruses may involve formation of defective hetero-oligomers of Rep proteins (Lucioli *et al.,* 2003). Too little is known, however, about oligomerisation of the nanovirus Rep, thus it is difficult to speculate whether the Rep of BBTV can oligomerise with Rep proteins from other viruses to confer resistance against them.

In contrast to pUbi-RepOnly-nos, silencing of the Rep gene did not seem to occur with pUbi-R.ORF-nos, in which the *ubi*1 promoter was directing over-expression of both the BBTV Rep and its internal U5 ORF. In cells that were co-bombarded with pUbi-R.ORF-nos and the 1.1mers of BBTV DNA-R and -C,

pUbi-R.ORF-nos was shown to enhance replication of BBTV 1.1mers. Furthermore, unlike pUbi-RepOnly-nos, pUbi-R.ORF-nos was able to initiate replication of DNA-C (Fig. 4-2; Table 4-2). These results indicate that U5 ORF, that was untranslatable in pUbi-RepOnly-nos but translatable in pUbi-R.ORF-nos, may encode a PTGS suppressor.

Suppressors of PTGS have not been found in nanoviruses, but have been characterised from several geminiviruses. For example, the AL4 genes (sometimes also known as AC4 or C4) of several geminiviruses encode suppressors of PTGS (Chellapan *et al.,* 2004; 2005; Vanitharani *et al.,* 2005; Bisaro, 2006). The PTGS suppressor encoded by the AL4 gene can bind to single stranded siRNA to interfere with formation of RISC and block events that follow (Bisaro, 2006). Similar to the U5 ORF of BBTV, the AL4 gene is often located internal to the Rep gene in geminiviruses (Bisaro, 2006). Amino acid sequences encoded by U5 ORF of BBTV do not share however, any similarity with the AL4 proteins (Beetham *et al.,* 1997). As such, U5 could be a new type of PTGS suppressor. To suppress PTGS, U5 ORF may have to be expressed at a similar level to the Rep gene. In cells co-bombarded with pUbi-RepOnly and the 1.1mer of BBTV DNA-R and -C, the limited amount of U5 gene product that could have been expressed by the promoter in the DNA-R was not sufficient to suppress possible PTGS of Rep. Each Rep transcript could be protected by one U5 protein because the data seems to suggested that Rep and U5 needed to be at similar concentrations in cells to prevent Rep proteins from being silenced.

To better understand the U5 ORF of BBTV, we also constructed a plasmid pUbi-IntOnly-nos, that had U5 ORF over-expressed by a strong *ubi*1 promoter.

We found that presence of pUbi-IntOnly-nos weakly suppressed replication of BBTV 1.1mers in banana embryogenic cells that were co-bombarded with pUbi-IntOnly-nos and 1.1mers of BBTV DNA-R and -C. PTGS suppressors of geminiviruses have been shown to cause developmental defects in plants (Chellapan *et al.,* 2004; Vanitharani *et al.,* 2005). Similarly, over-expression of U5 by pUbi-IntOnly-nos may have interrupted the PTGS mechanism needed for normal functioning of plant cells, thus indirectly suppressing replication of the BBTV genome that depends on many cellular factors.

In conclusion, plasmid pUbi-RepOnly-nos could be a suitable transgene to confer resistance against BBTV and closely related nanoviruses by triggering PTGS of the Rep gene. Compared with pUbi-RepOnly-nos, plasmid pUbi-IntOnly-nos represents a less ideal candidate to confer genetically engineered resistance against BBTV, because pUbi-IntOnly-nos can only weakly suppress replication of BBTV. However, because the U5 ORF of BBTV potentially encodes a PTGS suppressor, resistance conferred by pUbi-RepOnly-nos may be overcome by high levels of viral inoculum producing excessive amounts of U5 protein.

# CHAPTER 5

## EFFECT OF REP MUTANTS ON THE REPLICATION OF *BANANA BUNCHY TOP VIRUS*

# Abstract

# Abstract

The integral genome of *Banana bunchy top virus* (BBTV) has at least six circular, single-stranded (css) DNA components, known as BBTV DNA-R, -U3, -S, -M, -C and -N. BBTV DNA-R encodes a replication initiation protein (Rep), that is the only essential protein required for BBTV replication. To investigate use of mutated Rep genes for pathogen-derived resistance (PDR) against BBTV, highly conserved amino acid residues within ATPase motifs A, B and C were identified, and these were subjected to site specific mutagenesis, namely K187→M in motif A, D224→I in motif B or N268→L in motif C. These mutated Rep ORFs were cloned into maize ubiquitin (*ubi*1) promoter-nopaline synthase (*nos*) terminator cassettes. Plasmids containing replicating 1.1mers (greater-than-unit-length) sequences of DNA-R and –C were also constructed. Each of the "*ubi*1-mutated Rep-*nos*" constructs were co-bombarded with the plasmid clone of DNA-C 1.1mer into banana cell suspensions, and results indicated that none of the Rep mutants could initiate *in vivo* replication of DNA-C. The "*ubi*1-Rep mutant-*nos*" constructs were also co-bombarded with plasmid clones of both the DNA-R 1.1mer and -C 1.1mer into banana cell suspensions; the results showed that the Rep with K187→M or N268→L mutations, when over-expressed by the *ubi*1 promoter, suppressed replication of DNA-R and -C significantly in bombarded cells. These Rep mutants may have the potential, therefore, to confer banana plants with PDR against BBTV infection.

# Introduction

# Introduction

BBTV belongs to the genus *Babuvirus* in the family *Nanoviridae*. The BBTV genome consists of multiple circular, single-stranded (css) DNA components, each encapsidated individually in small isometric virions (18-20 nm) (Burns *et al.,* 1994; 1995; Harding *et al.,* 1991). The BBTV integral genome components, DNA-R, -U3, -S, -M, -C and -N, are found consistently in all geographical isolates of the virus (Burns *et al* 1994; 1995). Each cssDNA component is ~ 1 kb, and encodes one ORF, except for DNA-R that has two ORFs (Beetham *et al.,* 1997). The large ORF of DNA-R encodes a replication initiation protein (Rep) (Hafner *et al.,* 1997b), while the internal ORF encodes a protein (U5) of unknown function (Beetham *et al.,* 1997). The function of DNA-U3 is unknown, while DNA-S, -M, -C and -N encode the coat protein, movement protein, cell-cycle link (Clink) protein and nuclear shuttle protein, respectively (Wanitchakorn *et al* 1997; 2000). Replication of integral BBTV genome components can only be initiated by the "master" Rep (M-Rep) encoded by DNA-R (Horser *et al.,* 2001a). As such, DNA-R appears be the only BBTV genome component that is absolutely essential for BBTV replication.

The BBTV genome replicates by a rolling circle mechanism, presumably in a manner similar to other plant cssDNA viruses, e.g. the geminiviruses (Hafner *et al.,* 1997a). In the model proposed by Hastie (1998), during infection, the viral genome enters the nucleus and the host polymerase synthesises the second strand (minus sense) of the viral DNA. Double-stranded (ds) DNA acts as a template for

unidirectional transcription to produce the Rep, which site-specifically binds and nicks at the apex (between the $T^7$-$A^8$ nucleotides) of the stem-loop structure in the viral DNA. After nicking, the Rep covalently binds to the 5' end, while positive sense ssDNA is synthesised from the free 3'-OH end to displace the 5' strand. After a full strand of ssDNA genome is synthesised, a new Rep cleaves and releases the newly synthesised ssDNA genome. The original Rep, which still binds to the 5' end of the ssDNA monomeric unit, ligates the two ends of ssDNA into the circular form.

The BBTV Rep is a multi-functional protein with a rolling circle replication (RCR) domain at the N-terminus and an ATPase domain near the C-terminus of the protein (Fig. 5-1) (Hafner *et al* 1997b). The RCR domain consists of RCR motifs 1, 2 and 3 (RCR-1, -2 and -3). The first ~30 amino acid residues of the Rep protein, including the RCR-1, form a β-sheet structure that is responsible for site specific binding to viral DNA (Vega-Rocha *et al.,* 2007). The ATPase domain consists of AAA+ ATPase (ATPase associated with various cellular activities) motifs A, B and C (ATP-A, -B and -C) (Gorbalenya *et al.,* 1989; Koonin, 1993). ATPase motifs are conserved in all the superfamily 3 (SF3) helicases, including the replication initiators of small DNA and RNA viruses (Gorbalenya *et al.,* 1989; Koonin, 1993; Clérot and Bernardi, 2006). In viral replication initiators like BBTV Rep, the ATPase domain may contribute to melting of the replication origin and unwinding of replicative intermediates. In geminiviruses, ATPase activity is essential for viral replication (Desbiez *et al.,* 1995).

BBTV Rep represents a potential target for development of genetically engineered resistance because it is the only viral protein essential for replication.

Use of a *trans*-dominant negative Rep mutant strategy to engineer virus resistance has been attempted with geminiviruses however, with only moderate success

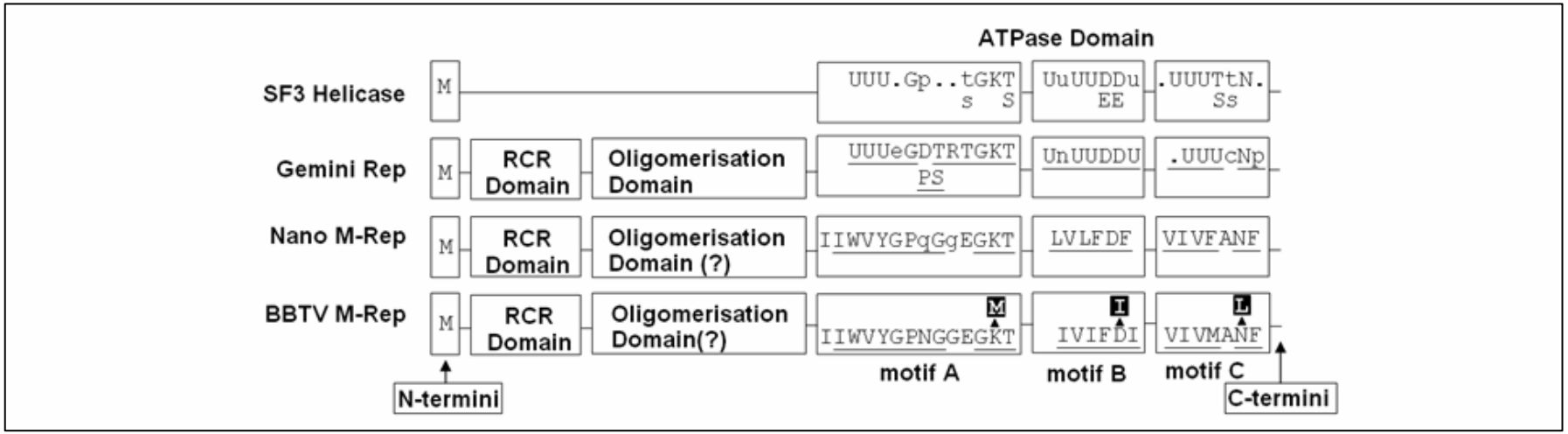



**Fig. 5-1. Schematic diagrams comparing the SF3 helicases, the (putative) Rep of geminiviruses, the M-Rep of nanoviruses and BBTV (Australian isolate; Accession No: NP_620727)**

Open box represents conserved domains or motifs. The sizes of boxes and spacing between boxes do not represent the actual sizes of each region. The consensus sequences of ATPase motifs A, B and C (modified from Koonin, 1993) are compared with the corresponding sequences in the M-Rep of BBTV DNA-R. The U represents bulky hydrophobic residues (ie. FILMVWY). The "." can be any residue. Upper-case amino acid abbreviations indicate the residue present in > 75 % of all aligned members; lower-case abbreviations indicate the residue present in > 50 % of aligned members (Koonin, 1993). The amino acid residues, which are consistent with the SF3 consensus sequences, were underlined. The ▲ and the shadowed amino acid abbreviations indicate the site-specific mutagenesis of this study. The oligomerisation domain has been observed in the Rep of geminiviruses, though an oligomerisation domain is suspected for BBTV Rep, the sequence within this region in the BBTV Rep (as well as the Rep of other nanoviruses) deviates from that of geminiviruses (data not shown).

(Shepherd *et al.,* 2007; Vanderschuren *et al.,* 2007). In this study, three of the conserved hydrophilic residues within the BBTV Rep ATPase motifs were mutated into hydrophobic residues (i.e. K187→M, D224→I or N268→L), and the interaction between these mutant Rep proteins and BBTV DNA-R and –C was analysed. The potential for these mutants to confer resistance against BBTV infection is discussed.

# Materials and methods

# Materials and methods

## *Generation of constructs*

### pBT1.1-R and pBT1.1-C

Constructs comprised 1.1mers of BBTV DNA-R (GenBank Accession No. NC_003479) and -C (GenBank Accession No, NC_003477), respectively, ligated into pGEM-T (Promega) (Fig. 5-2), and were generously provided by Dr. Cathryn Horser (Horser *et al.,* 2001a). Plasmids pBT1.1-R and pBT1.1-C were previously designated pBT1.1-1 and pBT1.1-5, respectively (Horser *et al.,* 2001a).

### pUbi-R.ORF-nos

The major ORF of BBTV DNA-R (Australian isolate; GenBank accession no. NC_003479) was amplified from pBT1.1-R using primers Bam_ORF1_F and Sac_ORF1_R (Table 5-1). The PCR mixture consisted of 10 pmol of each primer, 200 μM dNTPs, 1 U Expand DNA polymerase (Roche), and 0.1 μg of pBT1.1-R as the template (dissolved in sterilised $H_2O$) in 1x Expand PCR Buffer 1 (Roche). The mixture was heated at 92 °C for 2 min, followed by 35 cycles of 92 °C for 30 sec, 50 °C for 30 sec and 68 °C for 90 sec, followed by 1 cycle of 72 °C for 10 min. PCR product was ligated into pGEM-T (Promega) and transformed into *E. coli* DH5α using heat shock. Plasmids were extracted from selected clones, digested with BamHI and SacI enzymes, electrophoresed in a 1 % agarose gel in TAE buffer, pH 7.8, and stained with ethidium bromide. A DNA fragment of ~ 850 bp was excised and purified from the gel by a High Pure Gel Extraction Kit (Roche). This DNA fragment was inserted into the BamHI/SacI site between the maize polyubiquitin 1 (*ubi*1) promoter and nopaline synthase (*nos*) terminator, in

the plasmid pGEM-*ubi*-*nos*.

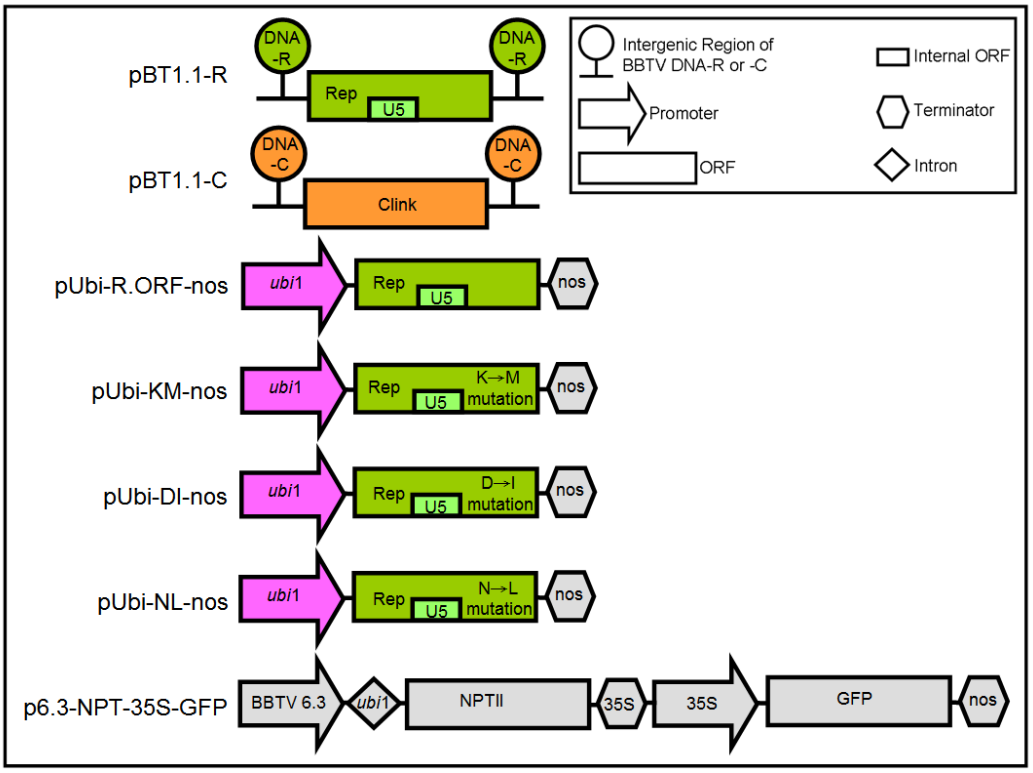



**Fig. 5-2. Maps of constructs**

The *ubi*1 promoter and intron are the promoter and intron of maize polyubiquitin 1 gene. The *nos* terminator is the terminator sequence of a nopaline synthase. The BBTV 6.3 promoter was derived from BBTV DNA-N (Dugdale *et al.*, 1998). The NPTII gene encodes the neomycin phosphotransferase II. The CaMV 35S promoter and terminator are the promoter and terminator sequences from *Cauliflower mosaic virus* (CaMV). The GFP gene encodes the green fluorescent protein from *Aequorea victoria*.

**Table 5-1. List of primers.**

| Name | Sequence* (5' to 3') | Location (nt) |
|---|---|---|
| ORF1F | ATGGCGCGATATGTGGTATGCT | 129-149 of DNA-R |
| ORF1R | TCAGCAAGAAACCAACTTTAT | 989-968 of DNA-R |
| BT5_240F | ATGGAGTTCTGGGAATCGTCTGCCATG | 240-266 of DNA-C |
| BT5_725R | TTAGAGTAATGTTACATCATAGTCTGA | 725-699 of DNA-C |
| Bam_ORF1_F | **GGATCC**ATGGCGCGATATGTGGTATGCT | 129-149 of DNA-R |
| Sac_ORF1_R | **GAGCTC**TCAGCAAGAAACCAACTTTAT | 989-968 of DNA-R |
| KM-F | CCCAAATGGAGGAGAAGGAATGACAACGTATGCAAAAC | 642-682 of DNA-R |
| KM-R | GTTTTGCATACGTTGTCATTCCTTCTCCTCCATTTGGG | 682-642 of DNA-R |
| TTP16 | CGAGGATATTGTTATATTTATTATTCCAAGATGCAAAGAGG | 753-793 of DNA-R |
| TTP17 | CCTCTTTGCAATCTTGGAATAATAAATATAACAATATCCTCG | 793-753 of DNA-R |
| TTP18 | GAAGTCATTGTAATGGCTCTCTTCCTTCCGAAGG | 886-919 of DNA-R |
| TTP19 | CCTTCGGAAGGAAGAGAGCCATTACAATGACTTC | 886-919 of DNA-R |

** The sequences of the restriction sites are shown in bold, while the sites of point mutations are underlined.*

**pUbi-KM-nos, pUbi-DI-nos and pUbi-NL-nos**

Constructs comprised three different mutations (K187→M, D224→I and N268→L) in the major ORF of BBTV DNA-R cloned into maize polyubiquitin (ubi1) promoter-nopaline synthase (nos) terminator expression cassettes. Mutations were introduced into the Rep ORF using a PCR-based method essentially as described in QuickChange Mutagenesis Kit (Stratagene), using pUbi-R.ORF-nos as the plasmid template and primer pairs KM-F/KM-R (for pUbi-KM-nos), TTP16/TTP17 (for pUbi-DI-nos) and TTP18/TTP19 (for pUbi-NL-nos). Primer sequences are shown in Table 5-1. The internal U5 ORF remained unchanged as in native DNA-R.

**p6.3-NPT-35S-GFP**

The construct (Fig. 5-2) was available from previous work and was generously provided by Mr. Matthew Webb (QUT). It comprised the BBTV 6.3 promoter-*ubi*1 intron-NPTII-CaMV 35S terminator together with the CaMV 35S promoter-GFP-*nos* terminator. This plasmid was used as a "stuffer" construct to ensure equal molar amounts of DNA were used in experiments. Further, the plasmid serves as a reporter gene (GFP) following microprojectile bombardment.

## *Sequencing constructs*

All constructs were purified using the BRESA-pure MAXi-prep Plasmid Purification kit (Geneworks). The constructs were sequenced using an automatic sequencer and Big Dye Termination Cycle Sequencing Ready Reaction V3.1 (PE Applied Biosystems). Primers used for sequencing included specific primers listed in Table 5-1 and M13 universal sequencing primers (US Biochemical).

## *Transformation by microprojectile bombardment*

Banana "Lady Finger" (*Musa* spp. AAB group) somatic embryogenic cell suspension cultures were prepared and maintained by Ms. Jennifer Kleidon and Mr. Don Catchpoole (QUT) as described by Khanna *et al.* (2004). The somatic embryos were harvested and approximately 0.1 g of condensed cell suspensions were plated onto filter papers and placed onto solid Bluggoe Low culture media (Dheda *et al.,* 1991). Each plate was bombarded with various combinations of constructs (1 μg each) using a particle inflow gun and gold microcarriers (BioRad) essentially as described by Dugdale *et al.* (1998).

Banana embryogenic cell suspensions were bombarded with the following combinations of plasmid constructs: (1) pBT1.1-C and pUbi-KM-nos; (2) pBT1.1-C and pUbi-DI-nos; (3) pBT1.1-C and pUbi-NL-nos; (4) pBT1.1-R and pBT1.1-C; (5) pBT1.1-R, pBT1.1-C and "stuffer"; (6) pBT1.1-R, pBT1.1-C and pUbi-KM-nos; (7) pBT1.1-R, pBT1.1-C and pUbi-DI-nos; (8) pBT1.1-R, pBT1.1-C and pUbi-NL-nos; (9) pBT1.1-R and pUbi-KM-nos; (7) pBT1.1-R and pUbi-DI-nos; and (8) pBT1.1-R and pUbi-NL-nos. To ensure an equal molar amount of DNA was co-bombarded each time, appropriate amounts of the stuffer construct p6.3-NPT-35S-GFP were also included where needed. On Day 4 or 8 post-bombardment, transformation efficiency was monitored by observing GFP expression in cells using a Leica MZ12 stereo microscope with GFP-Plus fluorescence module and green barrier filter (BGG22, Chroma Technology). Cell samples were also collected on these days. Cells from different plates were stored in Eppendorf tubes at -80 ºC prior to analysis.

### *DNA extraction*

Total nucleic acids were extracted from transformed and untransformed cells and dissolved in TE (pH 8), essentially as described by Stewart and Via (1993). RNA was removed by RNase A digestion and DNA concentration was quantified by spectrophotometry (Sambrook and Russell, 2000).

### *Generation of digoxigenin (DIG)-labeled probes by PCR*

The ORFs of BBTV DNA-R (DIG-ORF-R) and DNA-C (DIG-ORF-C) were used as probes. DIG-ORF-R was amplified from pBT1.1-R using primers ORF1F and ORF1R, while DIG-ORF-C was amplified from pBT1.1-C using primers BT5_240F and BT5_725R (Table 5-1). PCRs were done as described previously. PCR amplicons were electrophoresed through 1 % agarose gels in TAE buffer, pH 7.8, and stained with ethidium bromide. PCR amplicons of ~ 850 bp (DIG-ORF-R) and ~ 500 bp (DIG-ORF-C) were excised and purified from the gel using a High Pure Gel Extraction Kit (Roche), and used subsequently as templates for a second round of PCR, with the dNTP replaced with 5 µl DIG labeling mix (Roche) to incorporate DIG-label into the PCR products. The probes were purified using a QIAquick PCR purification kit and their concentration was quantified by spectrophotometry (Sambrook and Russell, 2000).

### *Analysis of transient transformants*

Replication and accumulation of BBTV DNA-R and -C in transformed cells was studied using Southern analysis. DNA was extracted from bombarded cell suspensions and 20 µg electrophoresed in an 1.5 % agarose gel in 1 x TAE buffer (pH 7.8) and stained with ethidium bromide. DNA (20 µg) from untransformed cell suspensions were included as a negative control. Size of DNA fragments on

agarose gels was determined using a DIG-labeled molecular marker III (Roche). Nucleic acids were transferred from agarose gels to positively charged nylon membranes (Roche) by 16 hours of capillary blotting (Southern, 1975). Membranes were baked at 80 °C for 2 hours, pre-hyrbidised in DI-Easy Hyb (Roche) at 42 °C for 1-2 hours, hybridised with 250 nmol of DIG-ORF-C in 10 ml of DIG-Easy Hyb at 42 °C for 12-16 hours, washed at high stringency (0.1 x SSC, 0.1 % SDS) at 65 °C prior to development as per the manufacturer's instructions (Roche), and exposed to X-Ray films (AGFA) for 30 min to 2 hours. Membranes were stripped as per manufacturer's instruction (Roche), hybridised with 250 nmol ORF-DIG-R, developed, and exposed to X-ray film again for 30 min to 2 hours. X-ray films were developed in an automatic developer (AGFA). Signal intensities on X-ray films were quantified using the densitometry function of TotalLab version 1.11 (Phoretix). The quantatative data was statistically analysed with type 3 of the 2-tailed *t*-test using Microsoft Office Excel 2003 version SP2 (Microsoft).

# Results

## *BBTV DNA-C replication was not initiated by pUbi-KM-nos, pUbi-DI-nos or pUbi-NL-nos*

To determine if replication of an integral BBTV genome component (DNA-C) could be initiated by over-expression of Rep mutants, pBT1.1-C was co-bombarded with either pBT1.1-R, pUbi-KM-nos, pUbi-DI-nos or pUbi-NL-nos. Cells were collected on Day 4 and 8 post-bombardment. Untransformed cells were also collected as a negative control. Total DNA was extracted from the cell samples, Southern blotted and hybridised with a probe specific to DNA-C. Replication was assessed qualitatively by presence of different conformational forms of BBTV genomic DNA including open circular, linear and supercoiled, in addition to multimeric intermediates that resulted from rolling circle replication. Identities of DNA conformations were based on reference to molecular weight markers and from previous studies (Horser *et al.,* 2001a). Replication was assessed quantitatively using densitometry readings based on supercoiled, replicative episomal forms of DNA-R.

In control cells co-bombarded with the plasmid clones of 1.1mers of DNA-R and -C and examined Day 4 and 8 post-bombardment, BBTV DNA-C, specific bands were observed on Southern blots, indicating replication of DNA-C had occurred (Fig. 5-3). DNA-C in the conformations of open circular, linear, supercoiled and various sizes of multimeric DNAs were observed. Replication of DNA-C was more abundant on Day 8 than on Day 4.

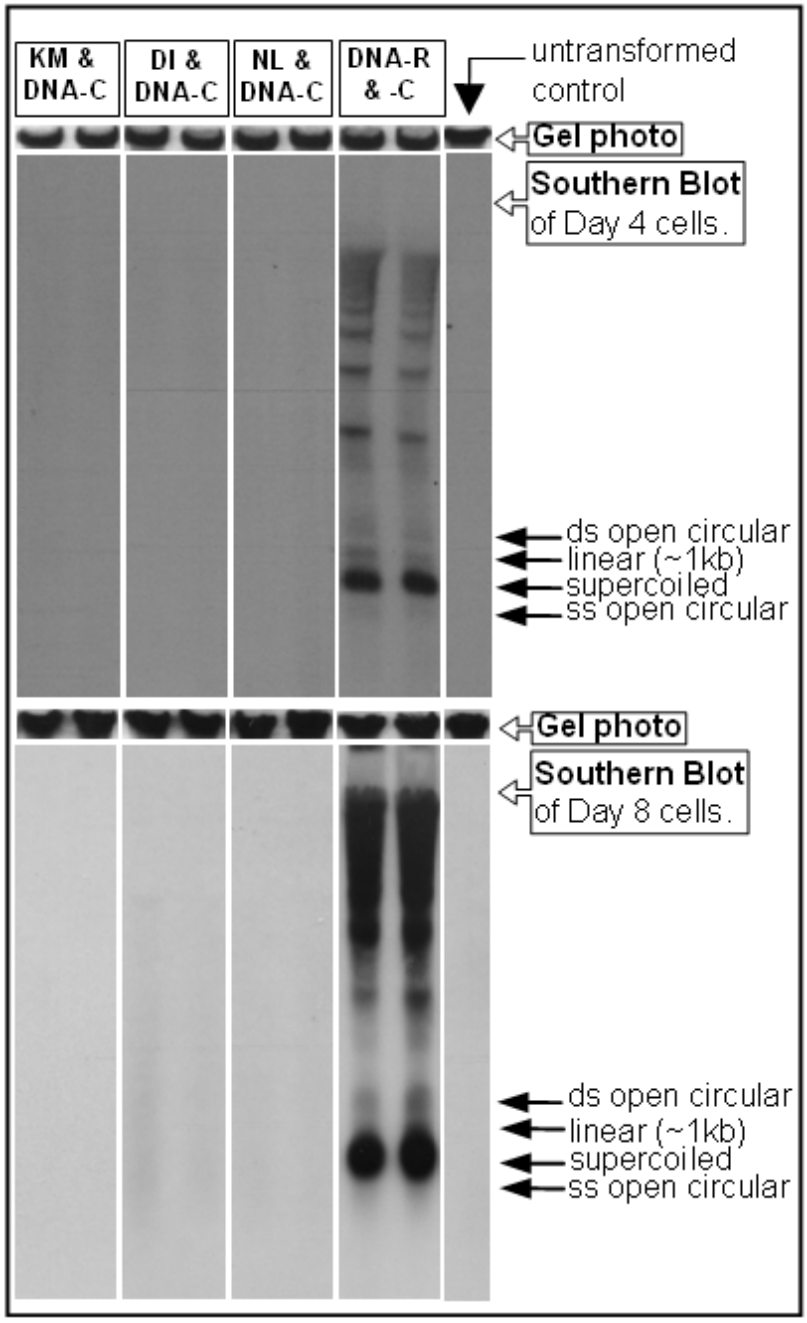



**Fig. 5-3. Replication of BBTV DNA-C in banana embryogenic cell suspensions**

Plasmids pUbi-KM-nos, pUbi-DI-nos, pUbi-NL-nos or pBT1.1-R were co-bombarded with pBT1.1-C into banana embryogenic cell suspensions. The replication of DNA-C in cells collected on Day 4 or 8 post-bombardment was studied by Southern analysis using probes specific to BBTV DNA-C. Blots were both exposed to X-ray films for 30 min. Sizes of the DNA molecules were estimated using DIG-labelled molecular marker.

In contrast, evidence of BBTV DNA-C replication (i.e. DNA-C in the forms of supercoiled DNA), was not detected in cells co-bombarded with pBT1.1-C and either pUbi-KM-nos, pUbi-DI-nos or pUbi-NL-nos, on either Day 4 or 8 post-bombardment (Fig. 5-3). Replication of DNA-C was not observed even after prolonged exposure (up to 24 hours) of Southern blots to X-ray films (data not shown).

### *pUbi-KM-nos and pUbi-NL-nos significantly suppressed BBTV replication*

To determine the effect of Rep mutants on replication of BBTV DNA-R and -C, plasmids pBT1.1-R and pBT1.1-C were co-bombarded separately and with either pUbi-KM-nos, pUbi-DI-nos or pUbi-NL-nos. Replication of DNA-R and -C in the cells was assessed by Southern analyses using probes specific to DNA-R or -C. Consistent with previous studies, control bombardments showed that DNA-R was capable of self-replication and that presence of pBT1.1-C enhanced DNA-R replication (Fig. 5-5 and 5-6). In cells co-bombarded with pUbi-KM-nos, pBT1.1-R and pBT1.1-C, DNA-R replication was significantly suppressed by presence of the K187→M mutant; DNA-C replication was only weakly suppressed on Day 4 (Fig. 5-4 and -5); but on Day 8, replication of both DNA-R and -C was nearly abolished in the presence of pUbi-KM-nos (Fig. 5-4 and -5). In cells co-bombarded with pUbi-NL-nos, pBT1.1-R and pBT1.1-C, replication of DNA-R and C was not affected by the presence of pUbi-NL-nos on Day 4, but interestingly, replication of both DNA-R and -C was nearly abolished by the presence of pUbi-NL-nos on Day 8. The pUbi-DI-nos construct did not have any significant effect on replication of DNA-R and -C on both Days 4 and 8.

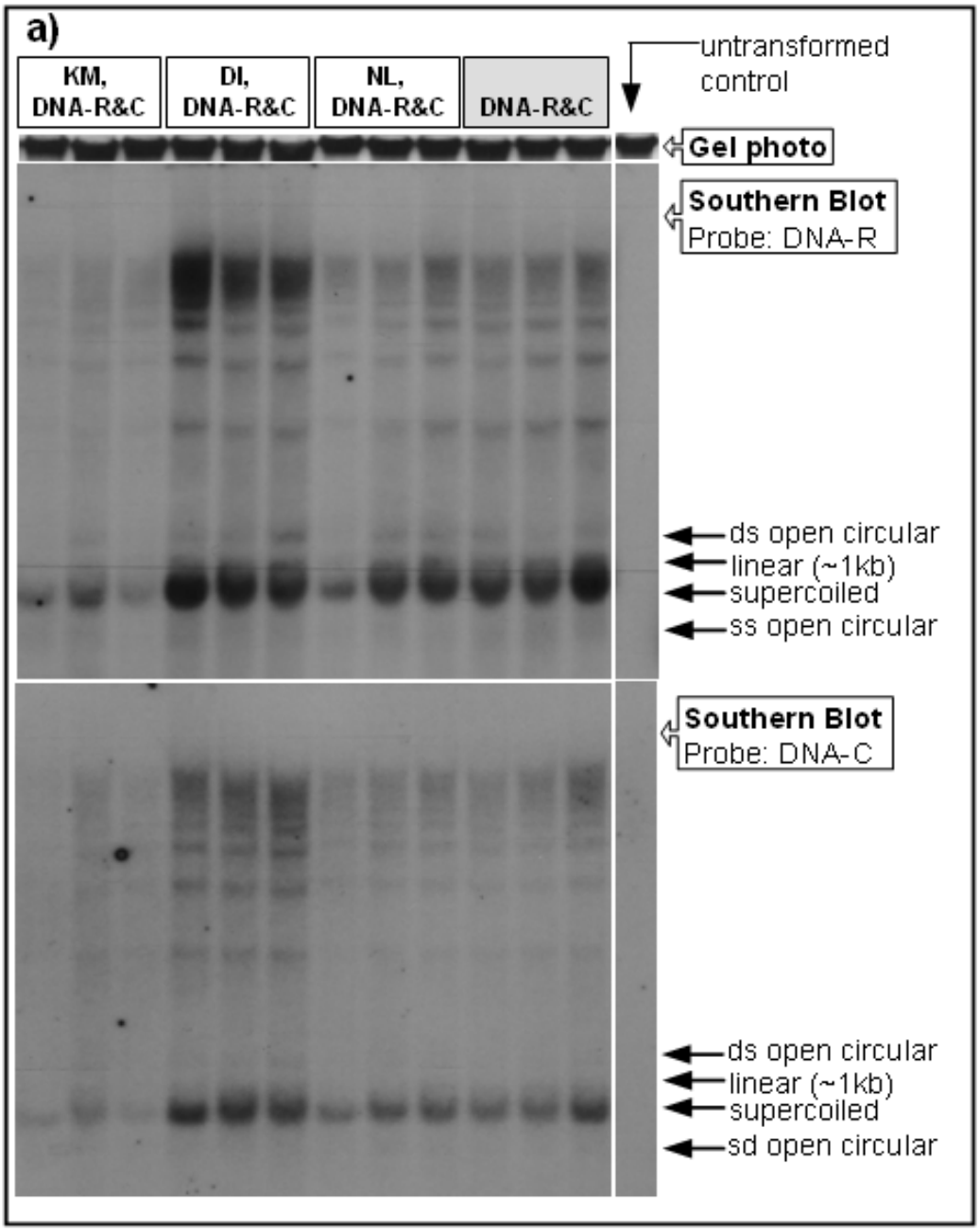



**Fig. 5-4. Replication of BBTV DNA components in banana embryogenic cell suspensions collected on Day 4 post-bombardment.** The pUbi-KM-nos, pUbi-DI-nos, pUbi-NL-nos or "stuffer" construct was co-bombarded with pBT1.1-R and pBT1.1-C into banana embryogenic cell suspensions.

**a)** The replication of BBTV DNA components was examined by Southern analysis using probes specific to BBTV DNA-R or -C. The blot was exposed to X-ray film for 30 min. Sizes of the DNA molecules were estimated using DIG-labelled molecular marker.

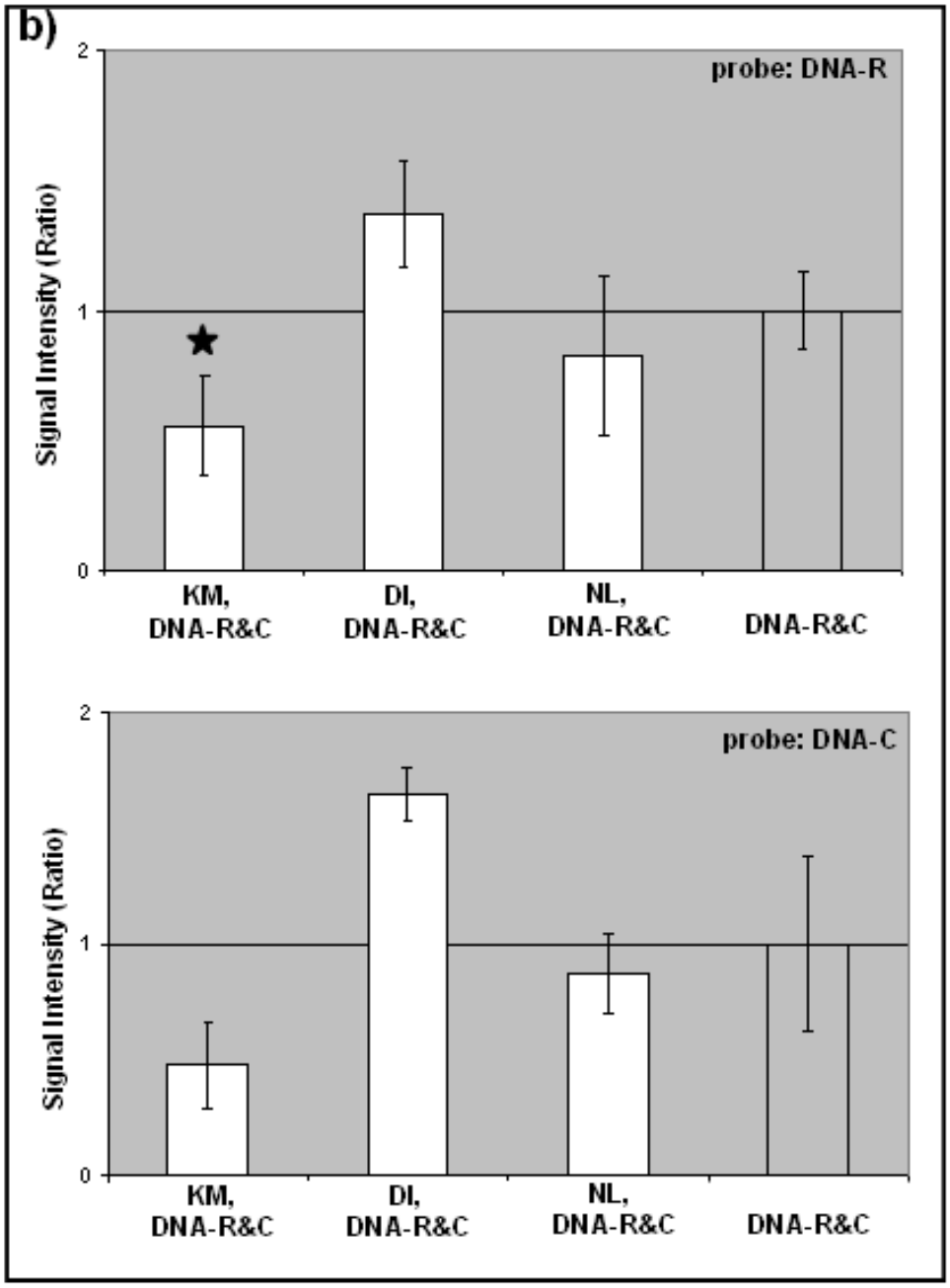



**Fig. 5-4. Replication of BBTV DNA components in banana embryogenic cell suspensions collected on Day 4 post-bombardment.**

**b)** Quantification of viral DNA. The amount of supercoiled DNA-R was quantified. The mean values are represented as bars for cells bombarded with different combinations of plasmids. The error bars indicated the 95 % confidence intervals. The replication of DNA-R was significantly suppressed ($P < 0.05$) by pUbi-KM-nos as indicated by the "★" sign.

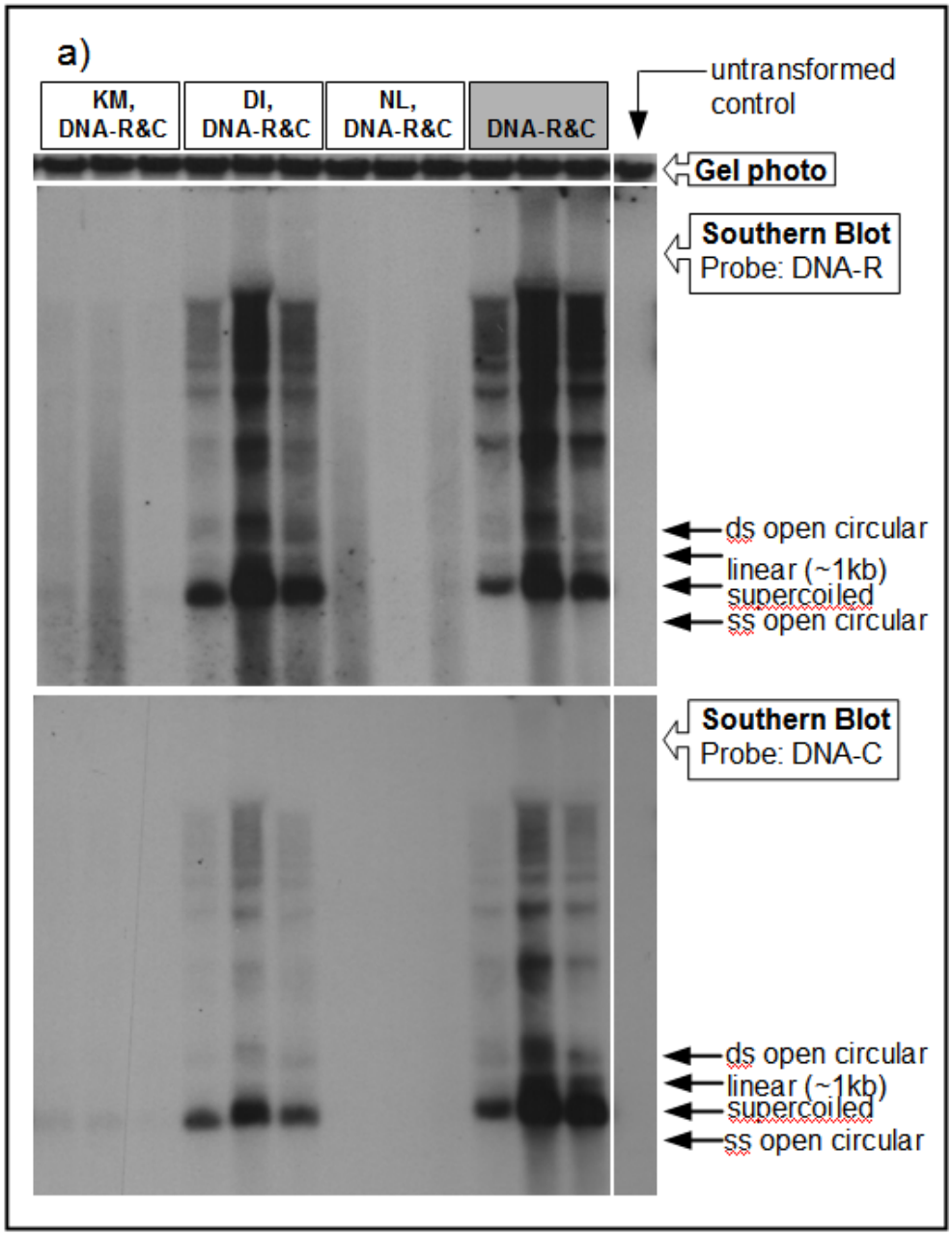



**Fig. 5-5. Replication of BBTV DNA components in banana embryogenic cell suspensions collected on Day 8 post-bombardment.** The pUbi-KM-nos, pUbi-DI-nos, pUbi-NL-nos or "stuffer" construct was co-bombarded with pBT1.1-R and pBT1.1-C into banana embryogenic cell suspensions.

**a)** The replication of the BBTV DNA components was examined by Southern analysis using probes specific to BBTV DNA-R or -C. The blot was exposed to X-ray film for 30 min. Sizes of the DNA molecules were estimated using DIG-labeled molecular marker.

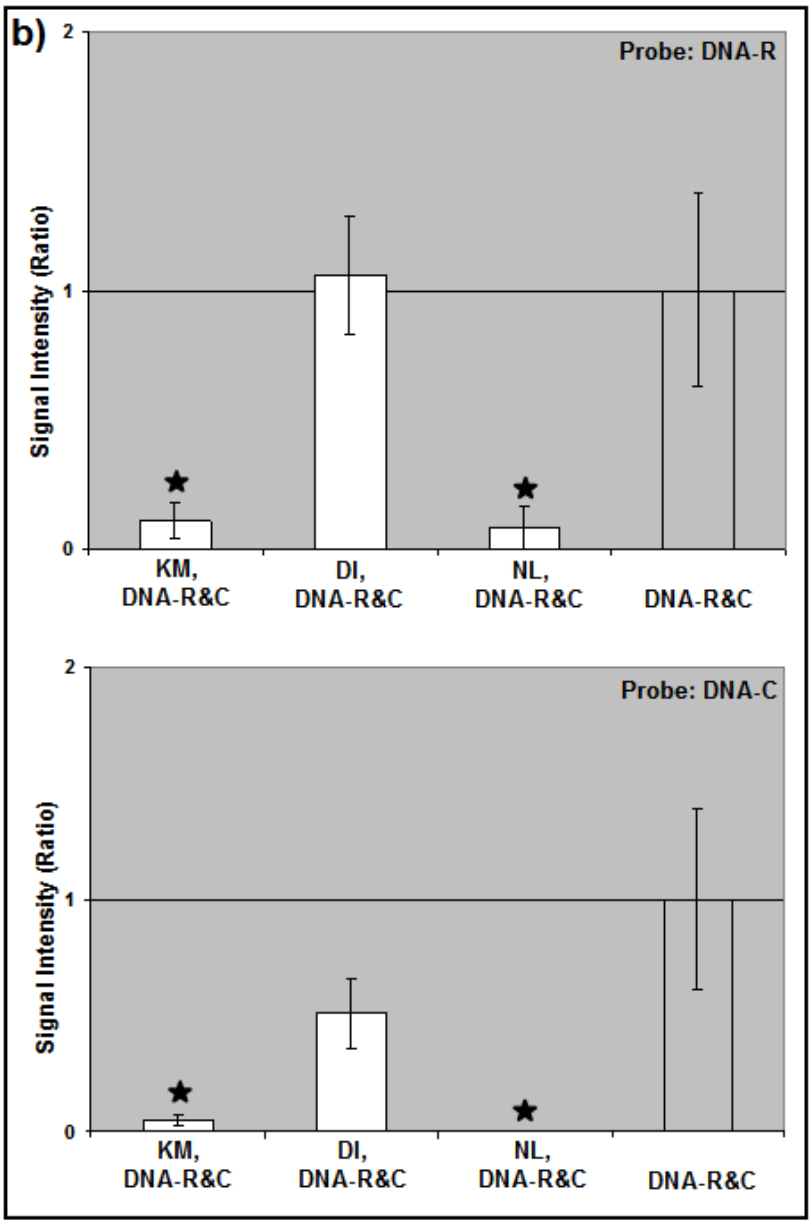



**Fig. 5-5. Replication of BBTV DNA components in banana embryogenic cell suspensions collected on Day 8 post-bombardment.** The pUbi-KM-nos, pUbi-DI-nos, pUbi-NL-nos or "stuffer" construct was co-bombarded with pBT1.1-R and pBT1.1-C into banana embryogenic cell suspensions.

**b)** Quantification of viral DNA. The amount of supercoiled DNA-R was quantified. The mean values are represented as bars for cells bombarded with different combinations of plasmids. The error bars indicated the 95 % confidence intervals. The replication of DNA-R and -C was significantly suppressed ($P < 0.05$) by pUbi-KM-nos and pUbi-NL-nos as indicated by the "★" sign.

The plasmids pUbi-KM-nos, pUbi-DI-nos, pUbi-NL-nos were also co-bombarded with pBT1.1-R into banana embryogenic cell suspensions, that were collected on Day 8 and assessed using Southern analyses (Fig. 5-6). Replication of DNA-R was almost undetectable in cells co-bombarded with pUbi-KM-nos or pUbi-NL-nos, while replication of DNA-R was not affected by pUbi-DI-nos. Results were similar to those obtained using DNA-C, only signals were generally weaker, suggesting DNA-R replicates less efficiently without DNA-C.

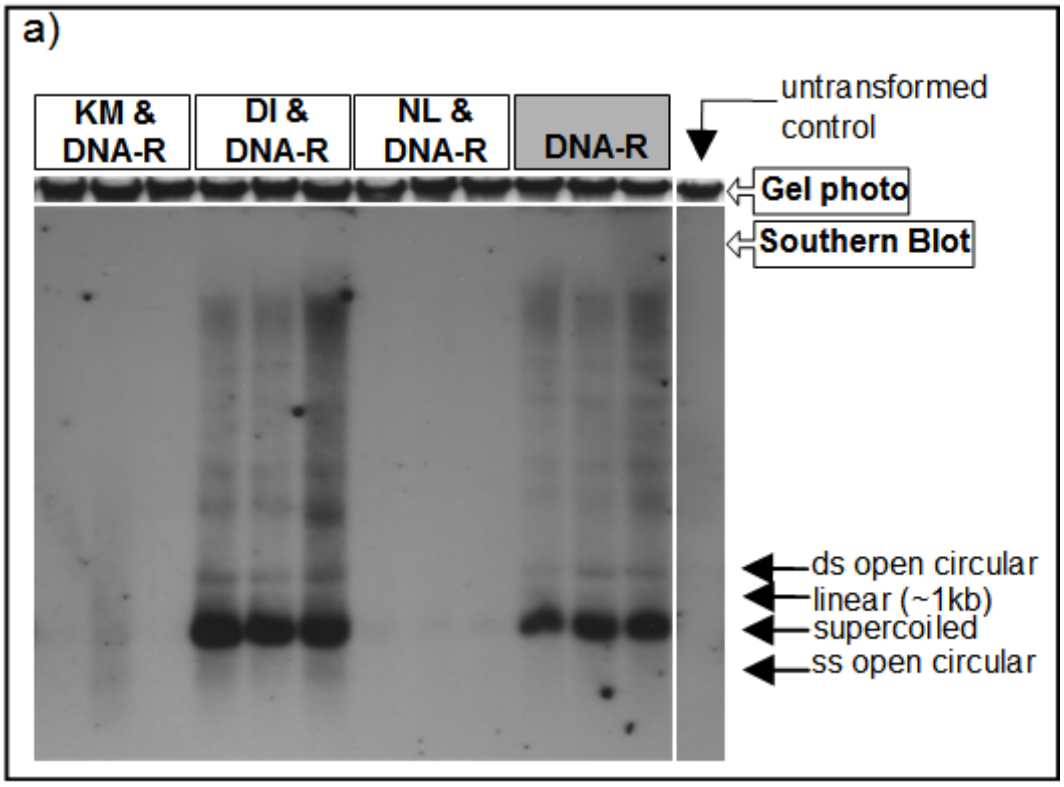



**Fig. 5-6. Replication of BBTV DNA-R in banana embryogenic cell suspensions.** The pUbi-KM-nos, pUbi-DI-nos, pUbi-NL-nos or "stuffer" construct was co-bombarded with pBT1.1-R into banana embryogenic cell suspensions.

**a)** The replication of the BBTV DNA-R was examined on Day 8 post-bombardment by Southern analysis using probes specific to DNA-R. The blot was exposed to X-ray film for 2 hours. Sizes of the DNA molecules were estimated using DIG-labeled molecular marker.

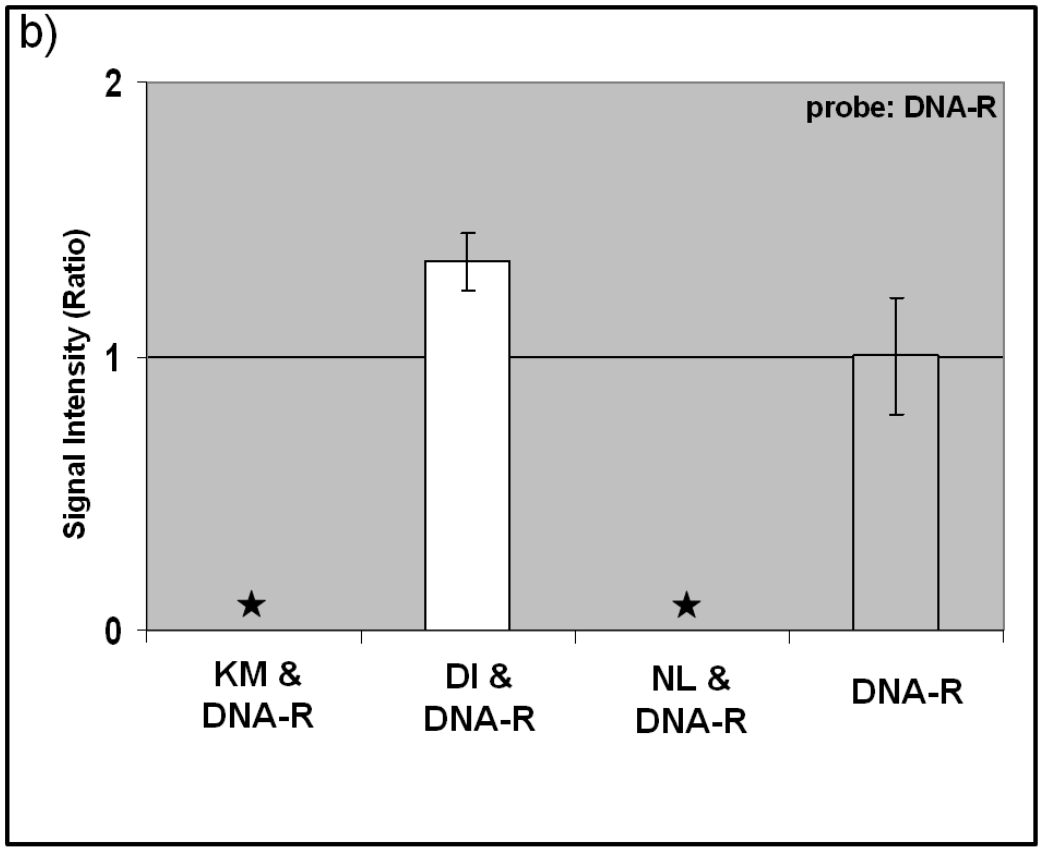



**Fig. 5-6. Replication of BBTV DNA-R in banana embryogenic cell suspensions.** The pUbi-KM-nos, pUbi-DI-nos, pUbi-NL-nos or "stuffer" construct was co-bombarded with pBT1.1-R into banana embryogenic cell suspensions.

**b)** Quantification of viral DNA. The amount of supercoiled DNA-R was quantified. The mean values are represented as bars for cells bombarded with different combinations of plasmids. The error bars indicated the 95 % confidence intervals. The replication of DNA-R was significantly suppressed ($P < 0.05$) by pUbi-KM-nos and pUbi-NL-nos, as indicated by the "★" sign.

# Discussion and conclusions

This study examined the effect of mutations in BBTV M-Rep on replication of BBTV. Ultimately, mutants that interfered with BBTV replication could potetially be used to engineer BBTV resistance in banana plants. Similar strategies have been used with some success in the geminiviruses. For example, plants transformed with Rep without functional ATPase motifs often show medium to high levels of resistance against the geminivirus from which the viral transgene had originated (Noris *et al.,* 1996; Brunetti *et al.,* 1997; Sangare *et al.,* 1999; Hanson and Maxwell, 1999; Polston and Hiebert, 2001; Polston *et al.,* 2001; Asad *et al.,* 2003; Antignus *et al.,* 2004; Hanley-Bowdoin *et al.,* 2004a; Chellappan *et al.,* 2004a; Yang *et al.,* 2004; Shivaprasad *et al.,* 2006; Shepherd *et al.,* 2007). Because M-Rep proteins of nanoviruses are related in sequence and biological functions to Rep proteins in geminiviruses and because ATPase activity is essential for viral replication (Vega-Rocha *et al.,* 2007), we hypothesised that a BBTV Rep that contained mutated ATPase motifs may confer resistance against BBTV.

In this study, K187→M, D224→I or N268→L mutations were introduced into the BBTV M-Rep to make the plasmids pUbi-KM-nos, pUbi-DI-nos and pUbi-NL-nos, respectively. The K187, D221 and N268 amino acids are highly conserved residues in the ATPase domain of SF3 helicases. The K187→M mutation was directed at ATPase motif A which has a consensus sequence of uuuxGpxg[ts]GK[TS] (Gorbalenya *et al.,* 1989). The targeted K187 residue of motif A is polar, positively charged, and usually hydrophilic in cellular conditions

(Livingstone and Barton, 1993). The K residue has been hypothesised to bind with the terminal phosphate group of a NTP molecule and form a complex with a $Mg^{2+}$ (Schlee *et al.,* 2001). Replacement of the M residue was non-polar, uncharged, and usually hydrophobic (Livingstone and Barton, 1993). As such, the K187→M mutation in this study may have disabled the NTP-binding ability of motif A, interfered with the protein quaternary structure and consequently disabled the ATPase/helicase activities of Rep. This was consistent with studies of Pause and Sonenberg (1992), Parsell *et al* (1994) and Choudhury *et al* (2006) (on a RNA helicase, a heat shock protein and a viral Rep, respectively), in which a K→A or K→E mutation within the ATPase motif A abolished helicase and ATPase activities and disturbed the quaternary structure of the protein.

The D224→I mutation was directed at ATPase motif B that is characterised by a stretch of hydrophobic amino acids, followed by one or two negatively-charged residues (i.e. D or E) (Gorbalenya *et al.,* 1989; Koonin, 1993). This motif chelates the $Mg^{2+}$ ion of the $Mg^{2+}$-NTP complex to stabilise binding of Rep to NTP (Gorbalenya *et al.,* 1989). The targeted D224 residue of the BBTV Rep is polar, negatively charged and hydrophilic (Livingstone and Barton, 1993). Replacement I residue is non-polar, hydrophobic, and aliphatic (Livingstone and Barton, 1993). Based on studies by Gorbalenya *et al.* (1989) and Choudhury *et al.* (2006), the D224→I mutation in this study is likely to affect ATPase/helicase functions of the Rep without disturbing quaternary structure of the protein.

The ATPase motif C is characterised by a hydrophobic stretch of amino acids followed by XXN (Neuwald *et al.,* 1999; Koonin, 1993). The conserved N residue is thought to interact with a water molecule that acts as the nucleophile in

ATP hydrolysis (Lenzen *et al.,* 1998). Hattendorf and Lindquist (2002) showed that a N→A mutation within the ATPase motif C of an ATPase (Hsp104) almost eliminated ATP hydrolysis, without disturbing NTP-binding ability. Replacement L residue was non-polar, hydrophobic and aliphatic (Livingstone and Barton, 1993). The N268→L mutation in this study could possibly eliminate the interaction between Rep and water molecules, and disable ATP hydrolysis required for ATPase/helicase activities.

When BBTV DNA-C was co-bombarded with pUbi-KM-nos, pUbi-KM-nos or pUbi-NL-nos, replication of DNA-C was not detected by Southern analyses in banana embryogenic cells collected on Days 4 and 8 post-bombardment. The K187→M, D224→I and N268→L mutations abolished ability of BBTV Rep to initiate BBTV replication.

The effect of the K187→M, D224→I or N268→L Rep mutants on replication of BBTV DNA-R and -C was also investigated. When DNA-R and -C were co-bombarded with either pUbi-KM-nos or pUbi-NL-nos, replication and accumulation of both DNA-R and -C was suppressed significantly.

The K187→M and N268→L mutants may have suppressed replication of BBTV genome components *via* a protein-mediated mechanism similar to that proposed by Brunetti *et al.* (2001). They transformed tobacco with a truncated Rep (without the ATPase domain) of TYLCV and obtained resistance to the virus (Brunetti *et al.,* 2001). Brunetti *et al.* (2001) showed that truncated Rep was still able to bind to the viral promoter but could not initiate replication (Brunetti *et al.,* 2001). As such, the K187→M and N268→L mutants presumably maintained

functional RCR domains that could recognise and bind specifically to the Rep promoter or stem-loop structure of integral BBTV genome components. The K187→M and N268→L mutants, which were over-expressed by the ubi1 promoter, may bind to the native Rep promoter in the DNA-R to suppress transcription of wild-type Rep gene (Castellano *et al.,* 1999). The limited amount of newly synthesised wild-type Rep may not be sufficient to initiate viral replication efficiently because the wild-type Rep would have to compete with the K187→M and N268→L mutants for substrates such as binding sites at the stem-loop structure of DNA-R and -C. It is also possible that the effect of the Rep mutations on BBTV viral replication was due to wild-type Rep also forming defective oligomers with the K187→M and N268→L mutants. Rep proteins of many viruses usually bind to DNA as monomers and then form double hexamers with other Rep proteins to initiate enzyme activities (Missich *et al.,* 2000).

Interestingly, the presence of pUbi-KM-nos almost abolished replication of DNA-R and DNA-C in co-bombarded banana embryogenic cells, as early as Day 4 post-bombardment, whereas the suppressive effect of pUbi-NL-nos was not observed on replication of DNA-R and DNA-C until Day 8. It is possible that pUbi-NL-nos suppressed replication less efficiently than pUbi-KM-nos because the N268→L mutation had less effect on the overall ATPase function of Rep. Unlike the K187→M mutation, the N268→L mutation was not expected to disturb the quaternary structure of the protein. Although the N268→L mutant was not expected to interact with water to support ATP hydrolysis, it is possible that when the N268→L mutant formed oligomers with wild-type Rep, the oligomers may still have normal Rep functions until accumulation of N268→L reached a certain threshold.

Suppression of BBTV DNA-R and -C replication by pUbi-KM-nos and pUbi-NL-nos was unlikely to involve RNA silencing of the Rep gene because the U5 gene, which was expressed as the internal ORF by both pUbi-KM-nos and pUbi-NL-nos, had been proposed to encode an RNA silencing suppressor (see Chapter 4). Also, if pUbi-KM-nos and pUbi-NL-nos could trigger RNA silencing of the Rep gene, pUbi-R.ORF-nos (i.e. a plasmid that could over-express both the wild-type Rep and internal U5 ORFs) would be most likely to also trigger RNA silencing of the Rep gene and suppress replication of BBTV genome components, because mRNAs produced by the above three plasmids had only one or two nt different in sequence and should behave similarly at the RNA level. Previous studies have shown however, that pUbi-R.ORF-nos did not suppress, but rather strongly enhanced replication of BBTV genome components (see Chapter 4).

The ratio of wild-type vs. mutant Rep needed to maintain the functions of Rep oligomers may depend on effect of the mutation. The pUbi-DI-nos did not suppress replication of DNA-R and DNA-C in transiently transformed banana embryogenic cells, possibly because the D224→I mutant may still be able to chelate $Mg^{2+}$ in conjunction with the native Rep in the oligomer, although less efficiently. Oligomers of Rep may not have lost function until the D224→I became more abundant so that oligomers consisted largely of D224→I mutants. Considering the *ubi*1 promoter is a strong promoter, the expression levels required for the D224→I mutant to suppress BBTV replication and confer resistance may never be achievable

It is important to note that the hypothesised oligomerisation-related effects of the Rep mutants on BBTV replication are based on the assumption that BBTV

Rep proteins function as oligomers on viral DNAs. Although this has not been reported for BBTV, oligomerisation of Rep of some nanoviruses has been observed (Vega-Arreguin *et al.,* 2005). The Rep of geminiviruses, as well as the Rep of many other small DNA viruses, function as double hexamers. Oligomers formed by Rep of nanoviruses may also be double hexamers. The amino acid sequences that were conserved in the oligomerisation domains of geminivirus Rep proteins were not found however, in the Rep proteins of nanoviruses (see Fig. 5-7 for the amino acid sequences of the M-Rep of nanoviruses; see Orozco *et al.* (2000) for sequences conserved in the oligomerisation domain of geminiviruses), suggesting that the Rep of nanoviruses may interact differently to form different oligomers.

The central region between the RCR and ATPase domains in the Rep of nanoviruses is assumed to be the putative oligomerisation domains based on analogies with the Rep proteins of geminviruses. Since the Reps of nanoviruses and geminiviruses have different amino acid sequences in their central regions, the central regions in the Rep of the nanoviruses may not be the oligomerisation domain.

Based on PDR-resistance studies on geminiviruses undertaken by Lucioli *et al.* (2003) and Chatterji *et al.* (2001), the spectrum of resistance against heterologous viruses would depend on ability of Rep mutants to form defective hetero-oligomers with the wild-type Rep of heterologous viruses. For BBTV M-Rep, precise location of the oligomerisation domain and the associated mechanism are not clearly understood. It is difficult to predict the breadth of resistance afforded therefore, by this strategy. Nevertheless, it may be reasonable

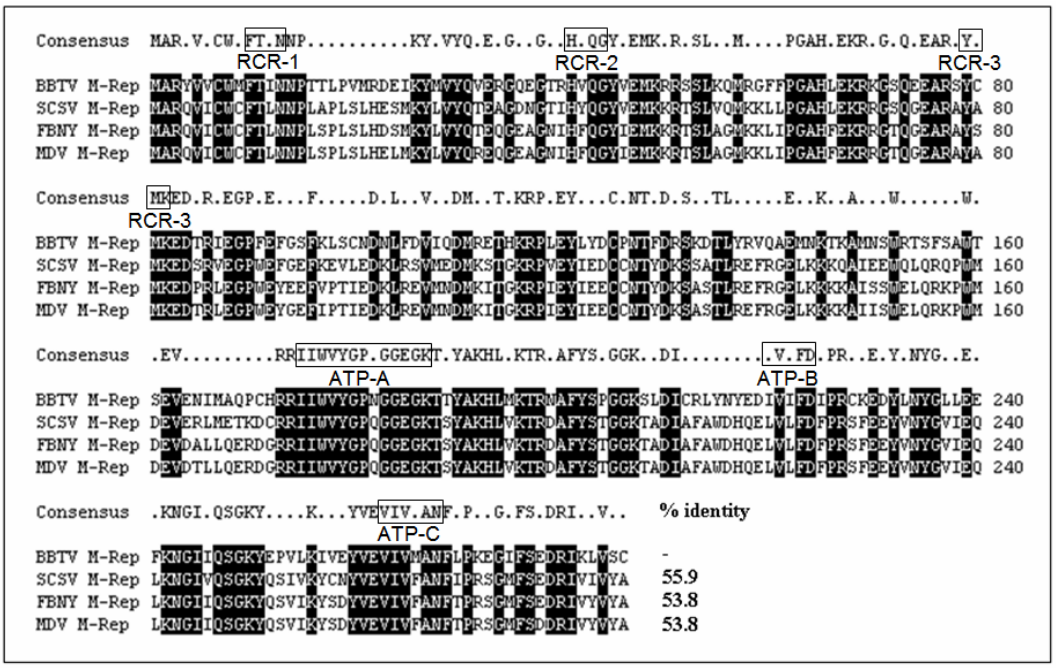



**Fig. 5-7. Amino acid sequence alignment of the M-Rep of nanoviruses.**

The amino acid sequences of Rep proteins of nanoviruses were aligned using the Clustal method, in DNAstar MegAlign software, with the residue weight table PAM100. The putative functional motifs, which were identified in reference to studies of Koonin (1993), are boxed.

Aligned sequences include the M- Rep of BBTV DNA-R (GenBank accession no. NP_604483), *Subterranean clover stunt virus* (SCSV; CAB96405), *Faba bean necrotic yellow virus* (FBNYV; Q9WIJ5) and *Milk vetch dwarf virus* (MDV; BAA97561). The percentages of identity to BBTV M-Rep are listed at the end of each sequence.

to assume that Rep with similar sequences, especially within the (putative) oligomerisation domain, will interact to form oligomers. The more than thirty BBTV M-Rep proteins characterised thus far, share > 92% homology in amino acid sequence, including the M-Rep proteins belonging to both the Asian and South Pacific BBTV isolates (Bell *et al.,* 2002; Furuya *et al.,* 2005). Therefore, the K187→M and D224→I mutants of the BBTV Australian isolate should be able to suppress replication of other geographical isolates of BBTV.

In summary, pUbi-KM-nos and pUbi-NL-nos both suppressed replication of BBTV genome components. Because suppression by pUbi-KM-nos was more efficient, however, pUbi-KM-nos could be a better candidate to generate a transgenic banana that is resistant to BBTV. Alternatively, the use of both pUbi-KM-nos and pUbi-NL-nos as transgenes may provide more effective resistance as it would necessitate the co-evolution of two different viral sub-sequences. The resistance mechanism is most likely to involve interfering with the wild-type Rep at the protein level, and not RNA silencing. The resistance spectrum is unknown, but should at least cover the different geographical isolates of BBTV.

# CHAPTER 6

# GENERAL DISCUSSION AND CONCLUSIONS

Banana is the most widely grown fruit in the world and is grown in more than 130 countries, mostly in tropical and subtropical regions (FAOSTAT, 2007). *Banana bunchy top virus* (BBTV) causes one of the most severe diseases of banana. Due to the importance of banana and the severity of the disease caused by BBTV, it is imperative that bunchy top disease be controlled.

In Australia, the spread of BBTV is currently controlled by following strict phytosanitary protocols (Dale, 1987). New banana plantations can only be propagated from virus-free banana suckers or corms that have been indexed for BBTV (Harding *et al.,* 1991; Hafner *et al.,* 1995). Although this has controlled BBTV in Australia, it is not a practical option for most banana-growing countries (Dale, 1987). Thus, new control strategies are needed for BBTV.

One strategy is to develop PDR, where plants are transformed with part of a viral genome to develop resistance against the virus from which the transgene is derived (Sanford and Johnston, 1985). It has been applied successfully in various crop plants to confer resistance against many RNA viruses and a few DNA viruses. However, there has been no report of PDR against BBTV or other nanoviruses; although there are several reports of PDR against geminiviruses that, like BBTV, are also circular, single-stranded (css) DNA viruses replicated by a Rep-mediated rolling-circle mechanism. The genes encoding Rep, coat proteins, movement proteins, nuclear shuttle proteins or replication enhancer proteins in geminiviruses have all been investigated for their potential to confer resistance against viruses, with Rep genes providing the best option (Vanderschuren *et al.,* 2007; Hanley-Bowdoin *et al.,* 2004a; 2004b). Hence, here we investigated the potential of the BBTV Rep to confer resistance against BBTV.

Various constructs of the Rep gene encoded by BBTV satellite DNAs and BBTV DNA-R were co-bombarded into banana embryogenic cell suspensions with 1.1mers of the BBTV integral genome components (i.e. pBT1.1-R and pBT1.1-C, which are plasmids consisting of 1.1mers of BBTV DNA-R and -C, respectively). Constructs that suppressed the replication of 1.1mers of BBTV integral genome significantly were deemed to be potentially useful transgenes to generate BBTV-resistant banana plants.

BBTV satellite DNAs encode Rep proteins capable of self-replication but that cannot initiate replication of BBTV integral genome (Horser *et al.,* 2001a). The BBTV integral genome can only be replicated by the "master" Rep (M-Rep) encoded by DNA-R (Horser *et al.,* 2001a). Horser *et al* (2001a) also showed that 1.1mers of BBTV DNA-S1 suppressed, but did not abolish, replication of the 1.1mers of BBTV DNA-R and -C in co-bombarded banana embryogenic cell suspensions on Day 4 and 8 post-bombardment. Results presented have confirmed the results of Horser *et al* (2001a) but also showed that DNA-S1 could suppress replication of the BBTV integral genome as late as Day 16 post-bombardment. This suggests that the Rep encoded by DNA-S1 probably interferes with M-Rep to suppress replication of BBTV. To determine if additional suppression of BBTV replication could be achieved, plasmid pUbi-S1.ORF-nos, which consists of the strong maize ubiquitin 1 (*ubi*1) promoter expressing the Rep of DNA-S1, was constructed and used to examine the effect of over-expression of Rep on BBTV replication. Interestingly, instead of enhanced suppression, pUbi-S1.ORF-nos enhanced replication of the 1.1mers.

Furthermore, a BBTV satellite DNA, designated BBTV DNA-S4, was isolated and characterised from a Vietnamese BBTV isolate in this study. The plasmid pBT1.1-S4, that consisted of the DNA-S4 1.1mer, was constructed and co-bombarded with pBT1.1-R and pBT1.1-C into banana embryogenic cell suspensions. In contrast, to results seen with DNA-S1, DNA-S4 was found to enhance replication of the BBTV integral genome in bombarded cells. This result was consistent with occasional observations of severe disease symptoms in plants that were infected with BBTV isolates containing BBTV satellite DNAs (Su *et al.,* 2003). In addition, replication of a DNA-S4 1.1mer (in cell suspensions co-bombarded with pBT1.1-S4, pBT1.1-R, and pBT1.1-C) was found to be higher than DNA-S1 1.1mer (in cell suspensions co-bombarded with pBT1.1-S1, pBT1.1-R, and pBT1.1-C). It is possible that, although replicative BBTV satellite DNA can suppress BBTV integral genome by competing for resources required for replication, the Rep encoded by BBTV satellite DNAs can assist replication of BBTV. Hence, BBTV satellite DNAs and their encoded proteins are not considered to be ideal candidates for generating BBTV-resistant transgenic banana plants.

It is unknown how the Rep encoded by DNA-S1 and -S4 assisted replication of BBTV integral genome components. Horser *et al* (2001a) showed that DNA-S1 could only initiate self-replication, and not replication of the BBTV integral genome. In Horser *et al* (2001a), however, DNA-S1 Rep was expressed by a weak BBTV S1 promoter (Hermann *et al.,* 2001). The Rep specifically recognises iterons in intergenic regions of the genome of nanoviruses to initiate replication (Timchenko *et al.,* 2000; Arguello-Astorga and Ruiz-Medrano, 2001). Iterons in the BBTV integral genome have an organisation and sequence that is different to

iterons in BBTV satellite DNAs, therefore Rep DNA-S1 should not be able to initiate replication of BBTV integral genome. Recognition by interons however, may have become less specific when Rep was abundant, either because Rep was expressed by a strong promoter such as *ubi*1, or because there are more copies of the Rep gene as in the case of actively replicating DNA-S4. In addition, Rep proteins in geminiviruses have been shown to function as double hexamers and the Rep proteins of nanoviruses have also been suggested to function as oligomers (Orozco *et al.,* 2000; Vega-Arreguin *et al.,* 2005). The Rep encoded by satellite DNAs may assist replication of BBTV integral genome by forming functional hetero-oligomers with M-Rep, although oligomerisation of M-Rep and the Rep of satellite DNAs has yet to be studied.

The ability of M-Rep to suppress replication of BBTV was also investigated. It was found that the BBTV M-Rep gene has the potential to confer resistance against BBTV, because over-expression of M-Rep by the *ubi*1 promoter in plasmid pUbi-RepOnly-nos could completely abolish replication of 1.1mers of DNA-R and -C (in the cell suspensions co-bombarded by pUbi-RepOnly-nos, pBT1.1-R and pBT1.1-C). The pUbi-RepOnly-nos significantly suppressed the BBTV replication possibly because over-abundant Rep transcripts triggered post-transcriptional gene silencing (PTGS) of Rep, although this was not examined experimentally. Wild-type Rep genes in the anti-sense orientation have been reported to confer resistance against geminiviruses via PTGS of the Rep, but there has been no attempt to use sense-orientated wild-type Rep genes prior to the current study. Resistance against geminiviruses has been conferred by genes such as (1) the sense partial and anti-sense partial Rep sequence of *Cotton leaf curl virus* (CLCuV) (Asad *et al.,* 2003), (2) the sense partial, anti-sense partial and

anti-sense wild-type Rep of TYLCV (Bendahmane and Gronenborn, 1997; Yang *et al.,* 2004), and (3) the anti-sense wild-type Rep gene of ToLCV (Praveen *et al.,* 2005) and TGMV (Day *et al.,* 1991). Resistance that was demonstrated in these studies was strain specific because > 90% nucleotide (nt) sequence homology was required between the transgene and the targeted gene to trigger PTGS, with the exception of *African cassava mosaic virus* (ACMV) (Chellappan *et al.,* 2004). The nt sequence of the ACMV Rep is only 66-67 % identical to Rep genes in *East Africa cassava mosaic Cameroon virus* (EACMCV) and *Sri Lankan cassava mosaic virus* (SLCMV), but plants transformed with the full-length Rep with a defective ATPase domain were resistant to all three viruses (Chellapan *et al.,* 2004). In these examples in geminiviruses, evidence of PTGS such as (1) a high level of transcription, (2) a low level of mRNA accumulation of Rep genes, or (3) 21-22 nt small interfering RNA (siRNA) molecules homologous to part of the Rep genes, were observed in resistant transgenic lines.

Interestingly, when the internal ORF (U5) and the M-Rep of DNA-R were both over-expressed by the *ubi*1 promoter in the plasmid pUbi-R.ORF-nos, replication of the 1.1mers of DNA-R and -C were enhanced significantly (in the cell suspensions co-bombarded by pUbi-R.ORF-nos, pBT1.1-R and pBT1.1-C). The function of U5 is unknown, but it may encode a PTGS suppressor. The co-bombarded pBT1.1-R also encodes U5, but the limited amount of U5 expressed by 1.1mer may not be sufficient to suppress the PTGS of M-Rep in cell suspensions. PTGS suppressors have not been found in BBTV or other nanoviruses, but have been found in geminiviruses (Bisaro, 2006; Noris *et al.,* 2004).

The potential of mutated M-Reps to confer resistance to BBTV by protein-mediated mechanism was also investigated. Studies on geminiviruses have shown that truncated Rep (T-Rep, Rep without ATPase domain) can confer resistance against several geminiviruses, including TYLCSV (Brunetti *et al.,* 1997; Lucioli *et al.,* 2003), TYLCV (Noris *et al.,* 1996; Antignus *et al.,* 2004) and TGMV (Hanley-Bowdoin *et al.,* 2004). Protein-mediated resistance against geminiviruses is often unstable because geminiviruses can sometimes silence transgenes to overcome resistance (Noris *et al.,* 2004; Lucioli *et al.,* 2003; Brunetti *et al.,* 1997). The U5 encoded by BBTV, however, may be a PTGS suppressor that can ensure expression of mutated Rep transgenes. A putative defective interfering (DI) DNA has been found associated with a mild strain (TW4) of BBTV, and this DI DNA has a 556 nt deletion within the region encoding the ATPase domain (Su *et al.,* 2003). Full-length Rep mutants (with defective ATPase domain) of ToMoV, BGMV and *Bean golden yellow virus* have been shown to suppress replication of geminiviruses from which Rep genes had been derived (Stout *et al.,* 1997; Hanson and Maxwell, 1999). Full-length Rep mutants have not been used however, to generate geminivirus-resistant transgenic plants because the C-terminal half of Rep proteins in geminiviruses encode functional motifs (e.g. Clink and myb-like motifs) that could inhibit regeneration of transgenic plants from transformed calli or induce disease symptoms (Shepherd *et al.,* 2007). These motifs are absent in the Rep encoded by BBTV.

Thus, in this study, site-specific mutations K187→M, D221→I and N248→L were made to the BBTV M-Rep gene to produce plasmids pUbi-KM-nos, pUbi-DI-nos and pUbi-NL-nos, respectively. K187, D221 and N248 are residues found consistently in ATPase motifs A, B and C, respectively,

of Rep proteins in geminiviruses and nanoviruses. Results have shown that the mutations disabled BBTV M-Rep and the three constructs of mutated Rep proteins were unable to initiate replication of 1.1mers of BBTV DNA-C in co-bombarded cell suspensions. The plasmids, pUbi-KM-nos, pUbi-DI-nos and pUbi-NL-nos, were also co-bombarded with pBT1.1-R and -C into cell suspensions and replication of 1.1mers of DNA-R and -C were examined post-bombardment. Two plasmids, pUbi-KM-nos and pUbi-NL-nos, suppressed replication of the BBTV 1.1mers significantly. The mechanism of suppression was not examined. Suppression of BBTV replication however, was presumably achieved by interfering with BBTV replication at the protein level because the pUbi-R.ORF-nos, that expresses almost identical mRNA transcripts as these two constructs of mutated Rep, did not suppress replication with BBTV 1.1mers. As has been proposed for geminiviruses by Brunetti *et al* (2001), over-expressed K187→M and N248→L Rep mutants may bind to the promoter on DNA-R to suppress transcription of wild-type Rep. The limited amount of wild-type Rep may also have to compete with mutated Rep for substrates such as DNA binding sites or form defective oligomers with mutated Rep (Brunetti *et al.,* 2001; Lucioli *et al.,* 2003). In addition, the D221→I Rep mutant has no significant effect on replication of BBTV 1.1mers. The D221→I mutated Rep may have suppressed transcription of wild-type Rep from DNA-R. However, although the D221→I mutated Rep alone did not replicate DNA-C, it might could still remaining functions that would assist wild-type Rep to replicate BBTV 1.1mers. The studies outlined above on ATPase motifs A, B and C in the BBTV Rep, has improved our current understanding of Rep and other helicases.

In summary, plasmids pUbi-RepOnly-nos, pUbi-KM-nos and pUbi-NL-nos all showed potential as transgenes to generate BBTV-resistant transgenic plants. Although plasmids were constructed with M-Rep ORF of BBTV DNA-R from an Australian isolate, any resistance conferred by pUbi-RepOnly-nos may have broader applications. This is because the nucleotide sequences of all BBTV M-Rep genes characterised thus far show > 92% homology (Bell *et al.,* 2002; Furuya *et al.,* 2005), and to trigger PTGS usually only requires a minimum of 90% homology between the transgene and the target gene (Ritzenthaler, 2005; Chellappan *et al.,* 2004). In addition, T-Rep proteins in geminiviruses have been shown to confer broad-spectrum resistance against up to three species of geminiviruses *via* a protein-mediated mechanism (Lucioli *et al.,* 2003; Chatterji *et al.,* 2001). Therefore, plasmids pUbi-KM-nos and pUbi-NL-nos may also confer resistance against all geographical isolates of BBTV.

Future directions for study include; banana plants should be stably transformed with pUbi-RepOnly, pUbi-KM-nos or pUbi-NL-nos, and then challenged with various geographical isolates of BBTV. The stability and level of resistance should then be tested by challenging transgenic plants with various levels of inoculation by different aphid vector numbers or prolonged inoculation. Challenged plants should be assessed for any viral accumulation and observed for disease symptoms. The mechanism of resistance could be studied by investigating presence of siRNA molecules and levels of mRNA accumulation in the resistant lines of stable transformants by Northern analyses using probes homologous to the BBTV M-Rep gene.

Stable transformation of banana plants will require optimisation. Firstly, to suppress BBTV replication, results from the current study suggest that Rep and U5 transgenes have to be over-expressed using a strong promoter. The *ubi*1 promoter is one of the strongest promoters for expression of transgenes in plant cells (Gallo-Meagher and Irvine, 1993). The CaMV 35S promoter has been shown, however, to express transgenes more strongly than the *ubi*1 promoter in non-cereal monocotyledons (Kamo *et al.,* 1995; Wilmink *et al.,* 1995). The *ubi*1 promoters in pUbi-RepOnly, pUbi-KM-nos and pUbi-NL-nos could be replaced with the CaMV 35S promoter to enhance expression of transgenes. Secondly, the "Lady Finger" variety of banana was used as the target here; in future research, other commercially important varieties of banana, including Cavendish, should be studied. Lastly, instead of particle bombardment as used in the current study, transgenesis should be trialled using *Agrobacterium* transfection that is a more reliable transformation method, with higher transformation efficiency and stability, lower copy number and rearrangement of transgenes, simpler integration patterns and less undesirable silencing of transgenes (Travella *et al.,* 2005).

In conclusion, using wild-type and mutated (i.e. K187→M and N248→L) Rep genes of BBTV to generate stable transformants of BBTV-resistant banana plants appears to be a promising strategy for controlling the spread of BBTV. The mechanism is not fully understood, but the strategy could be applied to confer resistance against the nanoviruses for which Rep-mediated resistance strategies have yet to be developed.

# CHAPTER 7

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